1-1-2011

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Ercan Selcuk Unlu

FUNCTIONAL COMPLEMENTATION ANALYSIS OF FUNGAL *RTG2* HOMOLOGS IN *SACCHAROMYCES CEREVISIAE*

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Life Sciences/Genetics
in the Department of Biological Sciences

Mississippi State, Mississippi

April 2011

FUNCTIONAL COMPLEMENTATION ANALYSIS OF FUNGAL *RTG2*

HOMOLOGS IN *SACCHAROMYCES CEREVISIAE*

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Title of Study: FUNCTIONAL COMPLEMENTATION ANALYSIS OF FUNGAL *RTG2* HOMOLOGS IN *SACCHAROMYCES CEREVISIAE*

Pages in Study: 128

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Changes in gene expression in response to mitochondrial dysfunction are mediated by components of the retrograde signaling pathway. The mitochondrial signal is recognized and transferred to the nucleus by dynamic interactions between regulatory proteins Rtg2p, Mks1p and Bmh1p. Retrograde signaling genes have been well characterized in the budding yeast *Saccharomyces cerevisiae* but very little is known about the retrograde response of other fungi. To identify retrograde signaling proteins in other fungi, the protein sequence encoded by the *S. cerevisiae RTG2* gene was used to search for fungal homologs using NCBI BlastP and the T-Coffee Multiple Sequence Alignment program. We selected four species having uncharacterized ORFs with more than 66% amino acid identity to Rtg2p for further analysis: *Ashbya gossypii*, *Candida glabrata*, *Vanderwaltozyma polyspora* and *Kluyveromyces lactis*.

In *S. cerevisiae*, cells deleted for *RTG2* are glutamate auxotrophs, and have reduced expression of Aco1p and Cit2p proteins. To determine whether the putative

RTG2 genes we identified encode bonefide regulators of the retrograde response pathway, we used standard yeast genetic approaches and molecular biology tools to investigate their ability to complement the defects associated with the *rtg2*Δ mutant using our *S. cerevisiae RTG2* shuffle strain. We investigated functional roles of Rtg2p homologs by comparing Cit2p and Aco1p protein levels, glutamate auxotrophy, as well as analyzing the interaction between Rtg2p homologs and Mks1p. We also analyzed sensitivity of mutant strains under various stress conditions to address possible signaling cross talk between the retrograde signaling pathway and the TOR pathway.

Our data show that the fungal Rtg2p homologs from *C. glabrata*, *V. polyspora* and *K. lactis* are functional in mediating the mitochondrial signal through known components of the retrograde signaling cascade. Our immunoprecipitation data suggest that TOR and retrograde signaling may exhibit cross pathway activation under rapamycin treatment. We show that Mks1p, the negative regulator of retrograde signaling pathway is required for Cit2p expression under rapamycin treatment. Given that all Rtg2p homologs showed low affinity for Mks1p which was in turn paralleled by a higher affinity of Mks1p for Bmh1p suggests that Rtg2p may have an additional functional role in influencing the association of Mks1p with Bmh1p.

DEDICATION

To my grandparents, Hamdi and Firdevs Ünlü

"For everything in this life, for materialism, for spirituality and for success our true mentor is science. Seeking mentors other than science is blindness, ignorance and heresy. It is our duty to realize the evolutionary phases of science through every single minute of our lives and to follow their progress."

Mustafa Kemal Atatürk, 1924

Quotation is English translation of original Turkish version

ACKNOWLEDGEMENTS

My first and the most special gratitude is for my advisor and committee chairman Dr. Donna M. Gordon for all her intellectual support during my study. With her exceptional guidance and encouragement, I must be the luckiest PhD student having such a perfect mentor. I will always remember her honesty, friendship and her dedication to science and follow her path to achieve my goals in science.

I would also like to thank my other committee members Dr. Susan M. Bridges, Dr. David E. Peebles, Dr. Daniel G. Peterson and Dr. David J. Chevalier. Their support was incredible that leaded me throughout my PhD study and helped me to expand my scientific knowledge in different fields of life sciences.

I would like to thank my colleague Lakshmi Narayanan from Dr. Chevalier's lab for her friendship and intellectual support. I am so lucky to have her as a friend. I believe that she will be one of the recognized scientists in the future.

I would like to thank all my friends in Starkville. Dr. Erdem Topsakal deserves more than sentences to express my gratitude. He was an elder brother, a friend as well as a mentor in every era of my life in Starkville. My special thanks also go to my dear friends Dayna Ellis, Charles Emrick, Hüseyin Tunç, uşağum Celal Güngör, Dr. Tutku Karaçolak, Dr. Kemal Efe Eseller, Dr. Erdem Acar, Harun Pirim, Hasan Hüseyin Enes Okur, Hasan Cihad Tekedar, Ersan Eyiler and Seyit Yüzüak.

I would like to my send my gratitude to my old friends and colleagues, Gönensin Ozan Bozdağ from Izmir Institute of Technology, Ersin Akıncı from University of Minnesota and Alaattin Kaya from Harvard University for their infinite friendship and intellectual support. Here, I would also like to thank my former advisor Dr. Ahmet Koç from Izmir Institute of Technology for his continuous guidance and intellectual support.

It would not be possible for me to finish my PhD in USA without the financial support which was sponsored by Republic of Turkey, Ministry of National Education. I will always appreciate for the support of Turkish taxpayers and follow a scientific path to deserve their support.

The last but not the least, I thank my family for their endless love and being a part of my life.

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CHAPTER I

INTRODUCTION

Overview

Cellular signaling pathways are major mechanisms controlling diverse cellular functions. One specialized intracellular signaling pathway is referred to as intraorganellar communication which is defined as a signal transduction pathway that delivers a signal from one organelle to a second organelle producing a cellular response. Depending on the cell type, different interorganellar signaling pathways are present. Often the signal is released by mitochondria, chloroplast, or plastid and received by the nucleus. Studies aimed at elucidating the mechanisms connecting the signal between organelles and the nucleus are important as many cellular functions are regulated through altered nuclear gene expression. Organelle-nuclear signaling pathways are classified into one of two groups: Retrograde signaling defines signals from organelles to the nucleus; anterograde signaling defines signals from nucleus to organelles [1]. In many cases retrograde signaling pathways are synchronized with the corresponding anterograde signaling to control the final functionality of the signal. The best studied retrograde signaling pathways include signaling between the mitochondria, chloroplast, plastid or golgi apparatus and the nucleus. Retrograde communication is regulated via cascade based kinase/phosphatase activities which lead to changes in the phosphorylation status of regulatory proteins. This signal cascade ultimately results in altered gene expression

which functions to coordinate the metabolic activities involved in nitrogen metabolism, energy metabolism, stress control and aging.

Mitochondria

Mitochondria (*singular*, *mitochondrion*) are double membraned structures that contain their own DNA (mtDNA). The cellular functions of mitochondria vary depending on the species or cell type; however they are responsible for several essential metabolic activities including ion homeostasis, initiation of apoptosis, lipid and amino acid metabolism, and energy production. Ninety percent of a cells total energy is supplied by mitochondria in the form of ATP [2]. Mitochondria house the enzymes for two metabolic pathways used for energy production: the tricarboxylic acid (TCA) cycle and electron transport chain (ETC) [3].

Mitochondrial morphology

The two membranes of mitochondria contain different sets of proteins establishing different biological functions. This distinct organization establishes compartmentalization of diverse functions, the regulation/control of these functions and the maintenance of mitochondrial morphology. The two membranes divide mitochondria into four separate compartments: *inner membrane*; *outer membrane*; the space between inner and outer membrane, or *intermembrane space*; and the interior space bounded by the inner membrane, or *matrix*. Membrane structures embedded within the two membranes enable mitochondria to control the transport of small molecules across the membrane. The outer membrane is highly permeable when compared to the inner membrane as it is perforated by porins, which allow for the passive diffusion of

molecules smaller than 1,000 daltons. Because of porin, the ionic properties and small molecule composition of the intermembrane space is more similar to the cytosol than the matrix. Unlike the outer membrane, trafficking of all materials across the inner membrane requires selective transport. The impermeability of the inner membrane maintains and controls the ion flow and membrane potential which is essential for several mitochondrial functions including ATP production and the initiation of apoptosis. The inner membrane is unique in that it forms invaginations called *cristea* which function to increase the area of the inner membrane surface thereby increasing the efficiency of energy production. By mass, seventy percent of the inner membrane is composed of protein including structural and antioxidant proteins as well as the super-complexes of the electron transport chain. The inner membrane also contains the specific lipid cardiolipin which is essential for regulating the stability of many inner membrane protein complexes [4]. Cardiolipin is also essential for maintaining the membrane potential which drives ETC [5].

Mitochondrial DNA

The functionality of the electron transport chain is dependent on intact *mitochondrial DNA (mtDNA)*. mtDNA is a circular double-stranded DNA molecule located in the matrix and averages 16 kilobases (kb) in humans, 80 kb in *S. cerevisiae*, and more than 200 kb in plants. The copy number of mtDNA varies between cells depending on environmental or growth conditions [6]. mtDNA encodes 2 rRNAs, 22 tRNAs and 13 polypeptides, and in yeast these 13 polypeptides are all subunits of the electron transport chain. Seven are subunits of complex I (NADH dehydrogenase), one is the cytochrome b subunit of complex III, three are subunits of complex IV (cytochrome c

oxidase), and two are subunits of complex V (ATP synthase) [7]. Therefore, the maintenance of mtDNA is essential for the functionality of the ETC. Yeast are unique however in that they can survive in the absence of functional mtDNA if provided with a fermentable carbon source. Under these conditions yeast lacking mtDNA are able to generate sufficient energy via cytosolic glycolysis. Because yeast cells lacking mtDNA are white in color due to the absence of heme and grow slower and form smaller colonies, they are referred to as *petite*. Petite cells can arise from the entire loss of mtDNA generating rho^0 cells, or the partial loss of mtDNA generating rho^- cells [4]. Petite formation is an irreversible process. Therefore, metabolic changes under these circumstances are crucial for cell survival. Mitochondrial retrograde signaling plays a crucial role in the survival of petite cells by changing cellular metabolism to favor the glyoxylate cycle over the TCA cycle for both energy production and metabolite biosynthesis (e.g. glutamate and lysine).

Mitochondrial retrograde signaling

In 1991, Liu and colleagues found that expression of *CIT2*, encoding the peroxisomal citrate synthase, was increased when cells were forced to form rho^0 petites [8]. This was the first study showing the involvement of mitochondrial retrograde signaling in the expression of metabolic genes in *S. cerevisiae*. Microarray studies found that cells with dysfunctional mitochondria altered their metabolic profile similar to that of cells in diauxic shift or cells grown under glucose limiting conditions [9-10]. These changes in gene expression patterns were shown to be regulated by mitochondrial retrograde signaling, the Hap complex (global regulator of respiratory gene expression) and Msn2p-Msn4p (transcription factors for general stress response). Studies revealed

that genes coding for early stage TCA cycle proteins [ALD4, ACS1, YAT2, CRC1, CAT2, CIT1, IDH1 and IDH2] and glyoxylate cycle proteins [SPO1, PXA1, CAT2 and CIT2] are co-expressed upon activation of the retrograde signaling pathway [11-12]. These genes reveal different roles for retrograde signaling in controlling metabolism of cells with dysfunctional mitochondria.

Under normal growth conditions, TCA cycle genes are regulated by the Hap complex. The Hap complex includes Hap2p, Hap3p, Hap4p, and Hap5p polypeptide subunits. This complex functions as a transcription factor when cells are grown in nonfermentable carbon sources. The activation of Hap complex is controlled by the expression level of the complex subunits [13-14]. However, with mitochondrial dysfunction, expression of TCA cycle genes revert to retrograde signaling control. Loss of mitochondrial function results in a block in the TCA cycle at the conversion of succinate to fumarate (Figure 1). Under these conditions retrograde signaling functions such that the TCA cycle can continue to produce metabolic intermediates for biosynthetic reactions [12].

Mitochondria and the TCA cycle

The tricarboxylic acid (TCA) cycle, also known as the citric acid cycle or Krebs cycle, is the driving force for respiring eukaryotic cells producing required metabolites for energy production and biosynthetic reactions (Figure 1). In the TCA cycle, the energy stored in carbon bonds is transferred to NAD or FAD to produce NADH or $FADH_2$, respectively. NADH and $FADH_2$ act as electron donors to the ETC for the production of ATP. The TCA cycle is a series of 10 successive enzymatic reactions. The first metabolite of the cycle, citrate, is produced from oxaloacetate and acetyl-CoA by the

activity of the mitochondrial isoform of citrate synthase (Cit1p). Following the isomerization of citrate to isocitrate by Aco1p, isocitrate is converted to α-ketoglutarate by isocitrate dehydrogenase (Idh1p/Idh2p). The next steps of the TCA cycle involve the further oxidation of α-ketoglutarate to produce the final product oxaloacetate. The early steps are under the dynamic control of the Hap complex and the Rtg1p/Rtg3p complex (transcription factors involved in retrograde signaling). Depending on respiratory status, yeast cells use either one of these transcriptional complexes to maintain a production of α-ketoglutarate by controlling expression of genes involved in α-ketoglutarate production [12, 15]. Homeostasis of α-ketoglutarate is also essential for amino acid biosynthesis and nitrogen metabolism reactions. The retrograde signaling pathway not only controls α-ketoglutarate biosynthesis in mitochondria, but also has a role in inducing the peroxisomal β-oxidation pathway and glyoxylate cycle for production of acetyl Co-A and citrate used to support early TCA cycle reactions ultimately increasing the production of α-ketoglutarate. Thus, integration of different metabolic pathways (mitochondrial TCA cycle, peroxisomal β-oxidation and glyoxylate cycle) by components of the retrograde signaling pathway is essential for yeast cells to overcome the decreased production of energy and metabolites due to mitochondrial dysfunction.

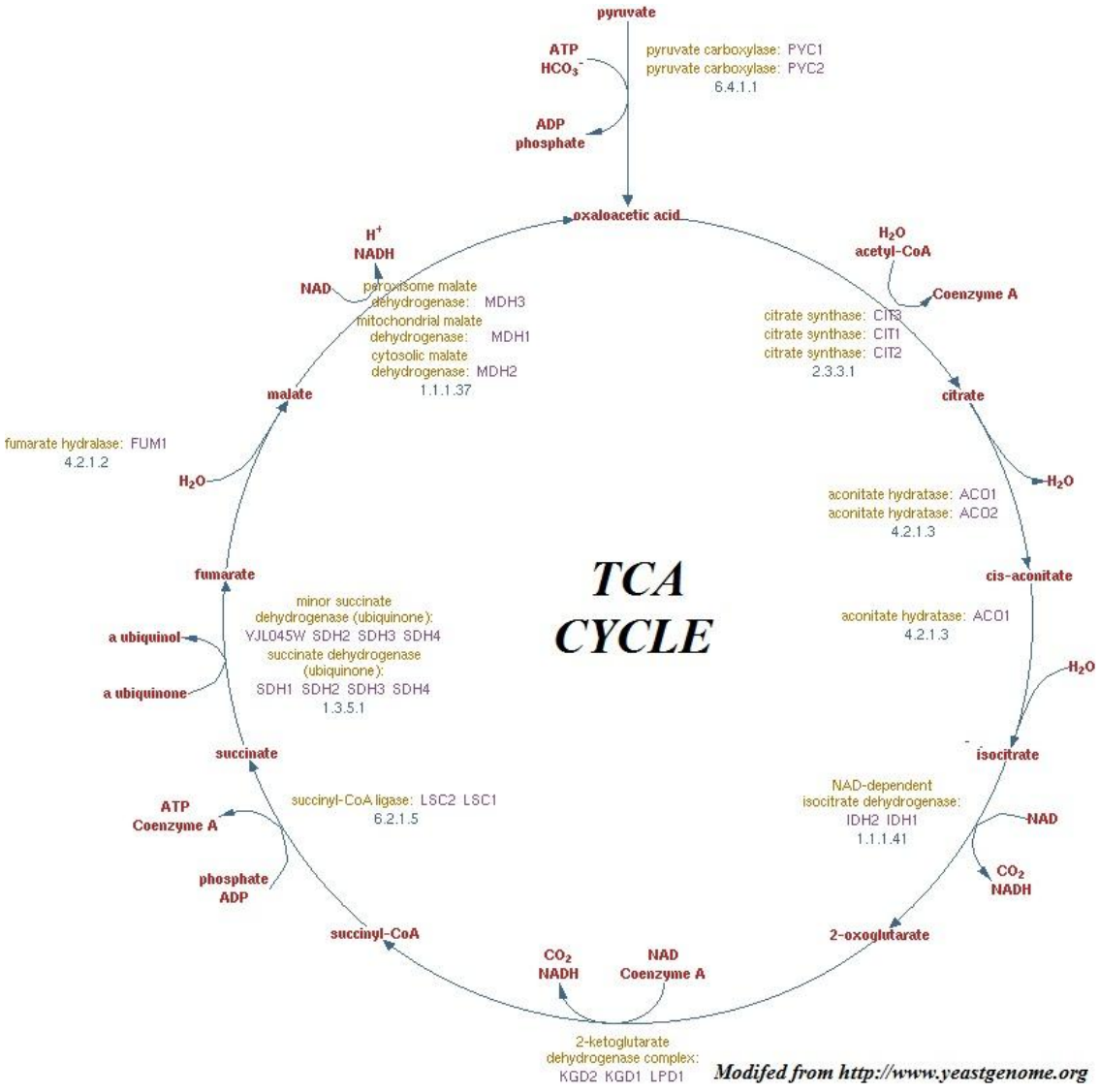


Figure 1. Tricarboxylic acid cycle

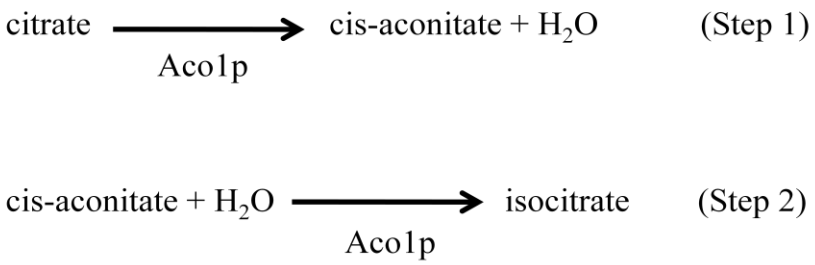
Diagram shows steps of the TCA cycle with yeast genes encoding the corresponding enzymes indicated.

Peroxisomes, glyoxylate cycle and α-ketoglutarate biosynthesis

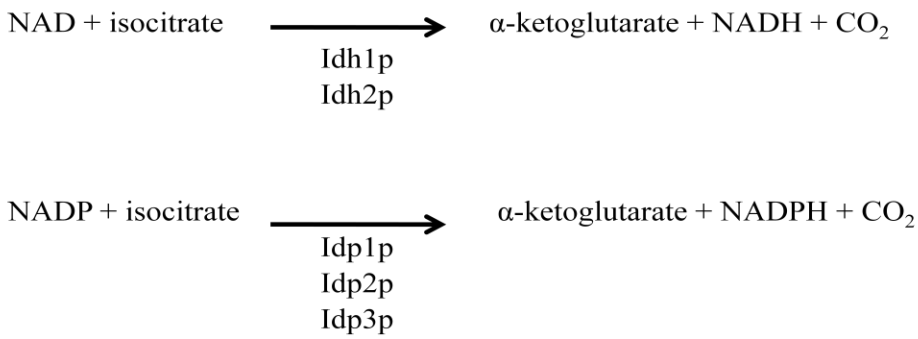
Peroxisomes are essential to eukaryotic cells as they are involved in the metabolism of amphipathic molecules and host enzymatic activities required for the metabolism of fatty acids, steroids, hydrophobic amino acids and certain purines [16]. Conjugation of β-oxidation via the glyoxylate cycle is a key feature of yeast peroxisomal metabolism allowing for growth in environments where fatty acids are the sole source of carbon. Unlike yeast, where the enzymes required for β-oxidation are localized to peroxisomes, in mammalian cells the enzymes for β-oxidation are located in mitochondria [17]. Regardless of location, a byproduct of the multi step β-oxidation pathway is acetyl-CoA which can be used by the cell for two purposes. Under starvation conditions, where fatty acids are available, acetyl-CoA is imported into mitochondria for energy production via the TCA cycle. In an alternative pathway, acetyl-CoA is processed by the peroxisomal glyoxylate cycle to generate succinate and glyoxylate. Succinate and glyoxylate are then used by the TCA cycle for energy production, or participate in biosynthetic reactions including amino acid biosynthesis, gluconeogenesis or feed-back loop control of the glyoxylate cycle. The final fate of these metabolites is determined by multiple factors as defined by the current environmental status of the cell.

The compartmentalization of the steps of the glyoxylate cycle to different organelles and the involvement of different enzyme isoforms function to increase the chance of survival of yeast when exposed to different environmental conditions (Figure 2). Similar to the metabolites succinate and glyoxylate, citrate is also utilized in different metabolic processes. In peroxisomes, citrate is generated from acetyl-CoA and

oxaloacetate by the peroxisomal isoform of citrate synthase, Cit2p [17]. Citrate is then converted to isocitrate by either the cytosolic or mitochondrial form of Aco1p. In yeast, both the mitochondrial and cytosolic forms of Aco1p are encoded by a single gene, *ACO1* [18].



The next step is oxidation of iso-citrate to α-ketoglutarate by isocitrate dehydrogenases [19-20]. α-Ketoglutarate has diverse roles in cellular metabolism ranging from energy production and amino acid biosynthesis to oxidative stress resistance. Homeostasis of α-ketoglutarate production must be maintained for the maintenance of these cellular processes. In *S. cerevisiae*, there are five isoforms of isocitrate dehydrogenase which are divided into two groups according to their requirements for either NAD or NADP. These five isoforms are localized to different subcellular compartments (mitochondria, cytosol and peroxisomes) and are all involved in α-ketoglutarate production.



The expression of mitochondrial NAD(+)-dependent isocitrate dehydrogenases (Idh1p/Idh2p) are regulated by the Hap transcriptional complex under robust respiratory conditions [21]. However, when respiratory activity is interrupted, the expression of *IDH1/2* reverts to control by the retrograde signaling pathway (Figure 3) [12]. Unlike Idh1p/Idh2p, production of α-ketoglutarate by NADP dependent isocitrate dehydrogenases (IDP1/2/3) is independent of respiratory competence. Instead, the supply of α-ketoglutarate by IDP1/2/3 is linked to carbohydrate biosynthesis [20, 22-24]. Each isoform of IDP supplements α-ketoglutarate levels in the mitochondria (Idp1p), the cytosol (Idp2p) or the peroxisomes (Idp3p) depending on cellular demand.

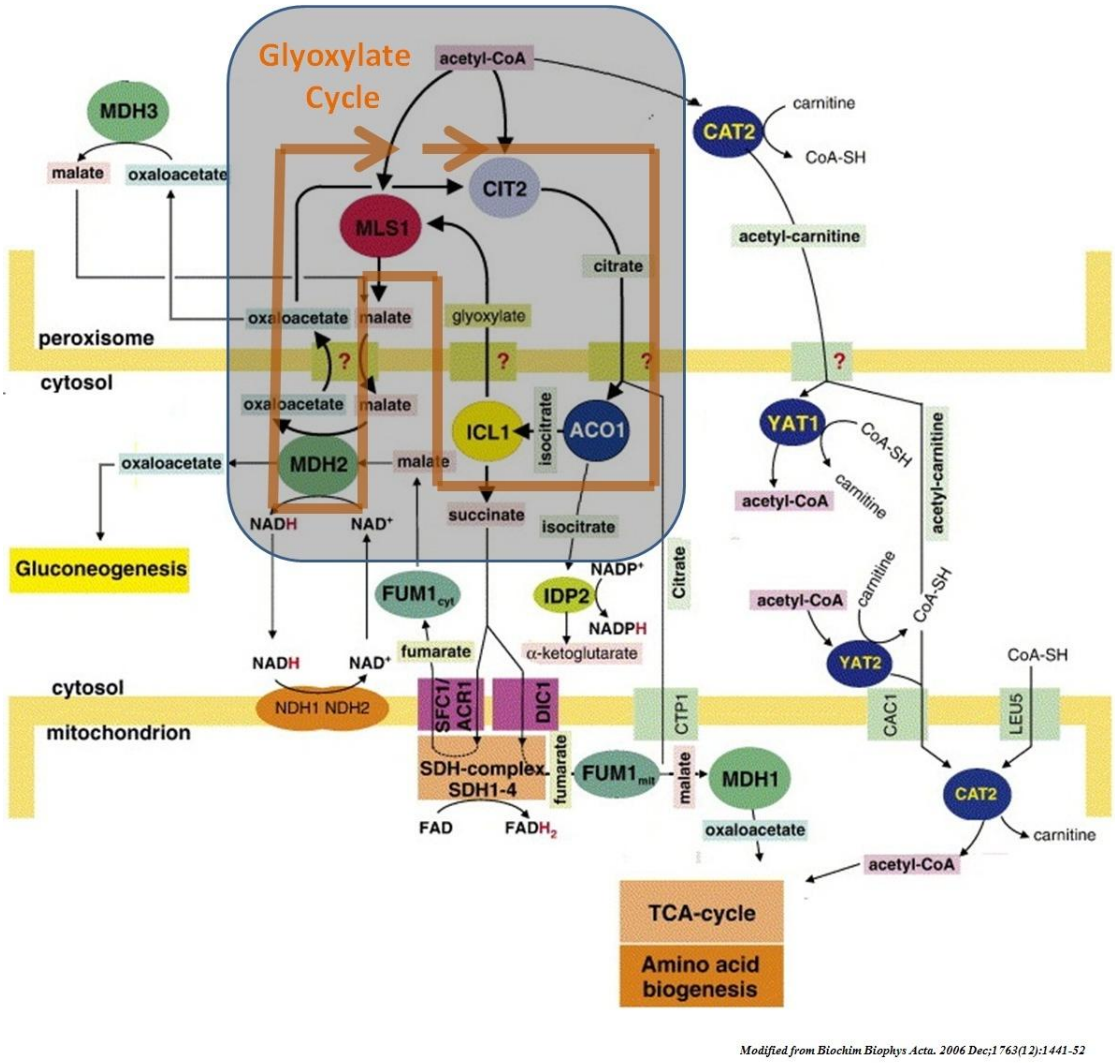
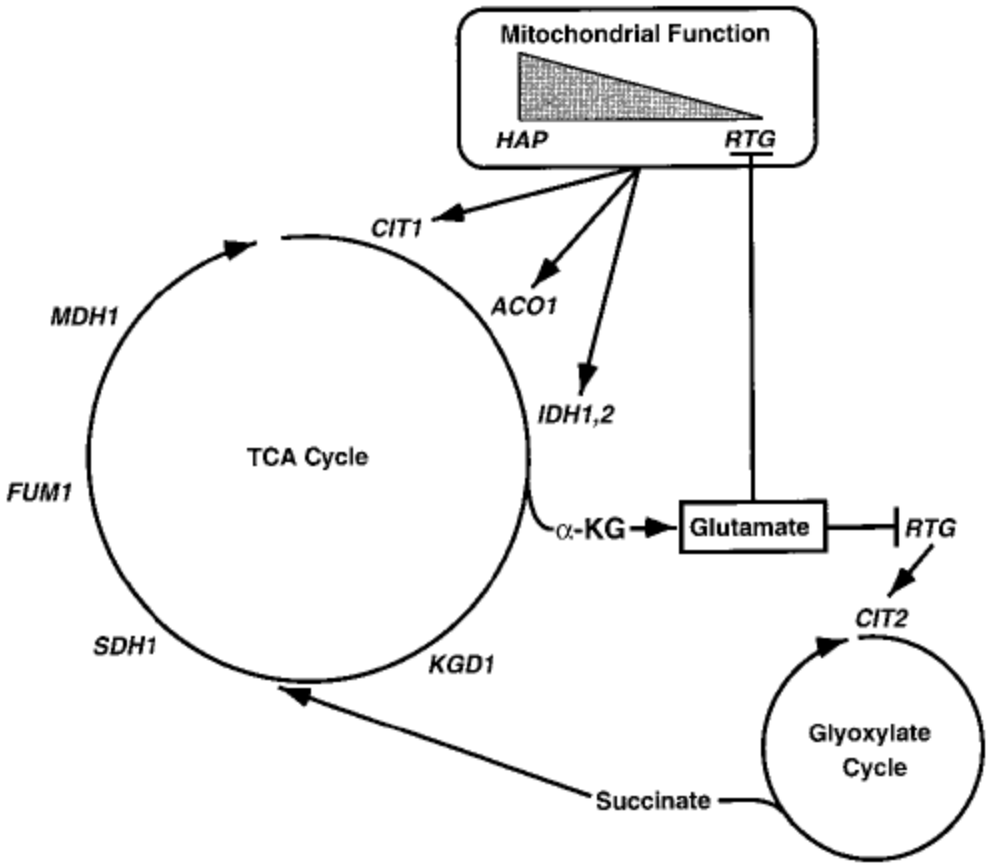


Figure 2. Glyoxylate cycle and its subcellular compartmentalization

Cartoon diagram depicting the glyoxylate cycle, the enzymes and their metabolites, along with their distribution within the peroxisome, cytosol and mitochondria. The overlapping metabolic activities carried out by the glyoxylate cycle, the TCA cycle and amino acid biosynthesis are shown.



Liu Z. and Butow R.A. Mol. Cell. Biol. 1999. 19(10):6720-6728.

Figure 3. Cross talk between the HAP and RTG1/3 complex

Cartoon diagram shows the transcriptional switch of the TCA genes between the HAP and Rtg1p/Rtg3p(RTG) complexes as a response to mitochondrial function [12].

α-Ketoglutarate is also involved in the metabolism of glutamate and glutamine. In yeast, the regulated expression of IDH and IDP control the level of α-ketoglutarate which indirectly influences the amount of glutamate and glutamine amino acid produced. These two amino acids are essential metabolites for nitrogen metabolism. Glutamate is synthesized by glutamate synthase (Glt1p) and glutamate dehydrogenases (Gdh1p and Gdh3p). Glt1p, the NAD(+)-dependent glutamate synthase, produces glutamate from glutamine and α-ketoglutarate in mitochondria; whereas the NADP(+)-dependent glutamate dehydrogenases, Gdh1p and Gdh3p, produce glutamate from ammonia and α-ketoglutarate in the cytosol and mitochondria, respectively. Production of glutamate from glutamine or ammonia by different enzyme isoforms is essential for multi-layered control of glutamate/glutamine levels under different stress conditions. For example, Gln1p expression is regulated by the availability of nitrogen sources [25]; while expression of Gdh1p and Gdh3p is controlled by the type and availability of carbon source [26-27].

Glutamate biosynthesis is essential for cell survival and production is strictly controlled by multiple overlapping pathways that are able to compensate for the loss from one through activation of an alternate pathway. For example, single mitochondrial isocitrate dehydrogenase gene mutants do not show glutamate auxotrophy as the remaining IDH isoforms can compensate for the reduction in α-ketoglutarate production. However, the Δ*idh1*/Δ*idp1* double mutant is unable to grow in media lacking glutamate as the mutations are in two different pathways [28]. Similarly, single null mutants for *GLN1*, *GDH1* or *GDH3* do not exhibit a glutamate auxotroph phenotype; however the triple mutant is a strict glutamate auxotroph [27]. Additional studies are required to

distinguish independent functions for each gene product as well as their expressional regulation. Current studies show that they have a key role in glutamate biosynthesis with regard to energy production, carbohydrate and amino acid biosynthesis, and antioxidant capacity. Studies have showed that *rtg2*Δ mutants, which are defective in mitochondrial retrograde signaling, are strict glutamate auxotrophs suggesting that mitochondrial retrograde signaling has an essential role in the maintenance of the global glutamate pool. Studies have also shown that retrograde signaling controls the production of glutamate not only under respiratory incompetency by changing the expression of early mitochondrial TCA cycle and peroxisomal glyoxylate genes, but also under nitrogen repressing conditions [24, 29-30].

Nitrogen metabolism

The element nitrogen (N) is essential for the survival of all living organism as it is a basic building block of nucleic acids and amino acids. Although 70% of Earth's atmosphere is nitrogen, most organisms cannot use elemental nitrogen as a nitrogen source except for certain groups of bacteria. Eukaryotic organisms have therefore evolved systems for the intake and use of nitrogen available as nitrogen salts and organic compounds. Once transported into the cells, nitrogen salts such as nitrate, are converted into biologically important nitrogen sources such as ammonium and glutamine. As the reduction of nitrate into ammonium is an energy consuming process, yeast cells prefer to use alternative nitrogen sources such as glutamate, glutamine, proline or urea [31].

Glutamate and glutamine are central nitrogen sources for yeast and function as nitrogen donors for amino or amido groups in amino acids, nucleic acids and lipids [32-33]. Not all sources of nitrogen are equivalent in terms of metabolic activities. For

example, the nitrogen source glutamine, glutamate and asparagine are "good" nitrogen sources, whereas proline and urea are considered "poor" nitrogen sources. The presence of "good" nitrogen sources block the intake and processing of "poor" nitrogen sources via transcriptional regulation. However, in the absence of a good nitrogen source, a change in transcription occurs to allow for the use of other available nitrogen sources in a process referred to as *nitrogen catabolite repression* (NCR) [34]. Regulation of genes controlled by NCR are under the control of the GATA transcription factors Gln3p, Gat1p (Nil1p) and Dal80p. Gln3p and Gat1p positively regulate the expression of genes coding for proteins responsible for a) the transport of poor nitrogen sources into cells such as amino acid permeases (Gap1p, Put4p and Can1p); b) catabolism of nitrogen containing molecules (Dur1p, Dur2p, Put1p, Put2p, Dal1p and Dal2p); and c) biosynthesis of glutamine (Gln1p, Gdh1p and Gdh2p) [35]. Gln3p transcriptional activity is regulated through its interaction with cytosolic prion protein, Ure2p. [36-37]. When Gln3p interacts with Ure2p, Gln3p is retained in the cytosol and is therefore transcriptionally inactive. Disruption of the association between Ure2p and Gln3p results in the translocation of Gln3p to the nucleus leading to the transcriptional activation of genes containing a GATA binding domain in their promoter [36, 38].

TOR pathway and control of NCR

Control of nitrogen utilization and uptake is crucial for the maintenance of cellular metabolic activities and cellular structures. A constant nitrogen supply is maintained through either control of central nitrogen metabolism or nitrogen catabolic repression depending on the availability of nitrogen sources. Under certain conditions, multiple metabolic pathways are regulated simultaneously for the adaptation to a new

environment. Thus, higher eukaryotes have developed complex control mechanisms which involve the co-regulation of nitrogen metabolism along with other metabolic pathways.

In yeast, the TOR (Target of Rapamycin) pathway is a central coordinator for general adaptive response metabolism under nitrogen repression. Two distinct TOR complexes are required for full coordination. The two complexes, TORC1 and TORC2 are distinguishable based on their number of subunits, their sensitivity to rapamycin and their roles in cellular metabolism. The TORC1 complex is made up Tor1p, Tor2p, Lst8p and Kog1p while the TORC2 complex consist of Tor2p, Avo1p, Avo2p, Avo3p and Lst8p [39]. These two complexes have different central functions in cellular metabolism. TORC1 is linked to stress related pathways primarily by controlling transcriptional machinery. TORC1 stabilizes the association of the phosphatase 2A-associating protein (Tap42) and Type 2A-related serine-threonine phosphatase (Sit4p) under nutrient rich conditions [40-42]. However, under nitrogen starvation conditions, or with rapamycin treatment, Tap42p and Sit4p interaction is interrupted. Sit4p is responsible for the disassociation of Ure2p from Gln3p resulting in the activation of GATA mediated transcription leading to activation of genes responsible for utilization of poor nitrogen sources [38]. TORC1 also stabilize the interaction between Msn2p/Msn4p complex and 14-3-3 proteins (Bmh1p/Bmh2p) preventing the nuclear localization of Msn2p/Msn4p. Nuclear localized Msn2p/Msn4p functions as a transcription factor responsible for activation of genes under stress conditions including carbon limitation [43]. Unlike TORC1p, limited information is available for the function of TORC2 although it is known that TORC2p is required for polarization of the actin cytoskeleton during the cell

cycle which is mediated by MAPK pathway [44-45]. Unlike TORC1, TORC2 is unresponsive to rapamycin treatment. Rapamycin is an immunosuppressive antibiotic which is used to reduce cell proliferation in a variety of eukaryotic organisms. It forms a toxic complex with Fpr1p, a peptidyl-prolyl *cis-trans* isomerase. This complex inhibits TORC1 function binding to Tor1p [39].

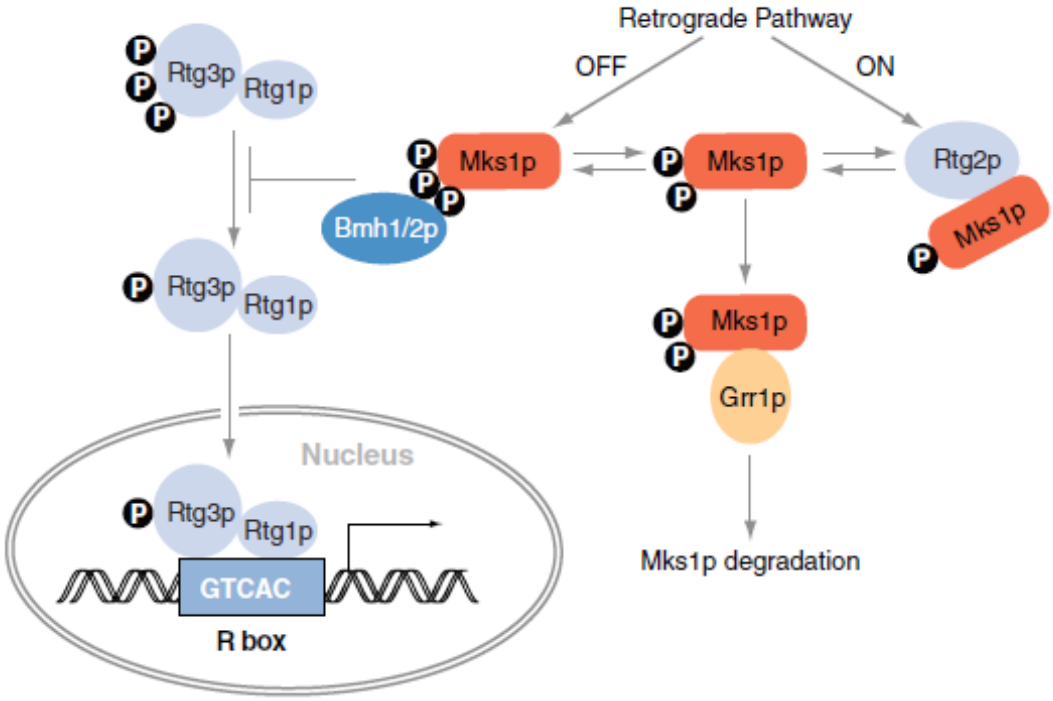
Recent studies have shown that in addition to GATA factor and Msn2p/Mns4p dependent gene regulation, TORC1p also affects the Rtg1p/Rtg3p dependent retrograde signaling pathway. For example, mutations in Lst8p, a subunit of the TORC1 complex, bypass the glutamate auxotrophy phenotype of *rtg2*Δ mutants suggesting that the TOR pathway has an inhibitory impact on mitochondrial retrograde regulation [29-30].

Yeast retrograde signaling

In general, cells must be able to respond rapidly to changes in intracellular and extracellular conditions. The mitochondrial retrograde signaling pathway regulates a cell's response to cellular stress, especially those that could lead to mitochondrial incompetency. Currently, the mitochondrial signal responsible for activation of this pathway is unclear; however glutamate levels have been shown to be critical for activation. The majority of the studies on yeast mitochondrial retrograde signaling have focused on the terminal processes of the pathway including the changes in gene transcription. However, very little is known about the upstream components of the pathway. In *S. cerevisiae*, the cytosolic protein Rtg2p is central to signal transmission as it is responsible for sensing the unknown signal released from dysfunctional mitochondria. Activation of the pathway is also dependent on the negative regulator, Mks1p. Note that when not bound to Rtg2p, Mks1p is either bound to the 14-3-3 protein

Bmh1p or degraded via regulated polyubiquitination [46-47]. The primary function of Bmh1p is to protect Mks1p from degradation while maintaining the inhibitory function of Mks1p. The binding of Mks1p to either Bmh1p or Rtg2p is regulated by the phosphorylation status of Mks1p with the dephosphorylated form of Mks1p having a high binding affinity for Rtg2p [46]. When Mks1p is bound to Rtg2p, the Rtg1p/Rtg3p heterodimeric complex is translocated from the cytosol into the nucleus where it acts as a transcription factor binding to promoter sequences carrying an R Box domain (GTCAC) [48]. This translocation event is mediated by dephosphorylation of the Rtg1p/Rtg3p complex. The proposed model for retrograde signaling activation by Liu and Butow is presented in Figure 4 as in their review article [11].

The genes induced by Rtg1p/Rtg3p code for proteins that participate in the TCA cycle and nitrogen metabolism [12]. This coordinated gene expression ensures that there is sufficient amount of α-ketoglutarate for biosynthesis reactions. One of the key genes upregulated by retrograde signaling in cells with dysfunctional mitochondria is *CIT2* which codes for the peroxisomal isoform of citrate synthase [8]. Cit2p functions in the glyoxylate cycle and is required for the conversion of oxaloacetic acid to citrate in peroxisomes. Citrate is transported into mitochondria where it is then used for the production of α-ketoglutarate, a main supply of nitrogen required for glutamate biosynthesis. As expected, mutations in *RTG2* result in the loss of Cit2p expression and glutamate auxotrophy. Given that the reduction in glutamate is an activator of retrograde signaling, it is likely that the levels of glutamate are a key regulator of retrograde activation [12, 15, 49].



Liu Z. and Butow R.A. Annu. Rev. Genet. 2006. 40:159-185.

Figure 4. Mitochondrial retrograde signaling
Cartoon diagram showing the known mitochondrial retrograde signaling pathway in *S. cerevisiae* [11].

Comparison to other organisms

The biological activities carried out by mitochondria are essential for all eukaryotic organisms including the control of mitochondrial competency via interorganellar signaling networks. Maintaining the functionality of mitochondria is dependent on retrograde mediated communication between the mitochondrion and the nucleus. Eukaryotic organisms can sense defects in respiratory competency due to mitochondrial DNA defects and respond via activation of retrograde signaling. However, except for one or two components of the signaling cascade, the molecular players and the genes controlled by retrograde signal vary between distant species or organisms.

In yeast, the downstream events in the retrograde signaling pathway have been characterized; however the signal delivered by dysfunctional mitochondria has yet to be identified. In contrast, in plant and mammalian cells it has been shown that Ca^{2+} plays an important role in the transduction of a retrograde signal from incompetent mitochondria, which along with a calmodulin dependent pathway, induce the expression of several mitochondrial proteins [24].

In mammalian cells, activation of the mitochondrial retrograde pathway occurs in response to the accumulation of mtDNA mutations, pathological conditions such as viral infections, and anoxic conditions and respiratory impotency. Mitochondrial membrane potential and integrity of membrane channels also play an essential role in the initiation of the retrograde signal. Under stress conditions depolarization of the mitochondrial membrane leads to the release of Ca^{2+} ions from mitochondria via ATP-dependent Ca^{2+} pumps [50-51]. Depending on cellular conditions and cell type, elevated cytosolic calcium triggers activation of the retrograde pathway that changes the expression of

proteins involved in the control of respiration, growth and development, apoptosis, inflammation, and immune response [52]. The calcium released by the mitochondria is recognized by different type of calcium sensitive factors including calcineurin protein kinase C (PKC), mitogen-activated protein kinase (MAPK), and Ca^{2+}/calmodulin-dependent protein kinase (CAMK) [53]. The presence of different sensory factors for Ca^{2+} dependent signaling is important for tissue specific response to mitochondrial retrograde signaling in mammalian cells. The tissue specific manner of the mitochondrial responses reflects the increased complexity of mitochondrial signaling pathways in higher eukaryotic organisms.

Like mammalian cells, mitochondria to nuclear signaling in plants is dependent on Ca^{2+} mediated events however, the pathway differs in its mechanisms of activation. In plants, stress conditions that activate mitochondrial retrograde signaling include both abiotic stress conditions (e.g. oxidative stress, cold and aging) and biotic stresses (e.g. plant-pathogen interactions) [54-55]. Typically, oxidative and aging based stresses are caused by the elevated production of reactive oxygen species which are alleviated through the production of enzymes involved in the reduction of reactive oxygen species (ROS) and the repair of damaged molecules. In the case of biotic stress, release of increased levels of salicylic acid, nitric oxide or reactive oxygen species have been reported to function as activators of cellular defense mechanisms. It has been suggested that the elevated ROS production under abiotic and biotic stress triggers the mitochondrial retrograde response [1, 56].

In plants, one of the genes upregulated by the mitochondrial retrograde signaling response pathway is *AOX1*a which encodes an alternative oxidase involved in the

alternative transport of electrons in the electron transport chain (ETC) [54, 57]. In a fully competent ETC, electrons are transferred from complex II to complex III oxidizing ubiquinol, and in the final step oxygen is reduced to water via oxidation of cytochrome c. In the alternative ETC pathway (AP), ubiquinol is directly oxidized by AOX1a to reduce oxygen to water. The basic function of the AP is to bypass an overloaded ETC by quick release of electrons. Transfer of these electrons through the alternative pathway can reduce ROS production, especially in cells inhibited at complex III [57].

The *AOX1a* gene contains a palindromic TGACG motif (G Box like domain) in its promoter that functions as a specific binding site for TGA1 protein, a bZIP transcription factor [54, 58]. Under pathogenic conditions, expression of the *AOX1a* gene is induced by binding of TGA1 to its promoter. This binding is dependent on interaction of TGA1 with NPR1 protein [54, 58-60]. NPR1, also known as disease resistance protein, interacts with bZIP transcription factors in the cytosol and nucleus, and is usually required for transcriptional activation under stress conditions. Upon phosphorylation of the TGA1/NPR1 heterodimer, the complex is translocated from the cytosol to the nucleus. Once in the nucleus, TGA1 binds to the G box like domain of *AOX1a* activating the transcriptional expression [59]. Interestingly, the interaction of TGA1 with NPR1 has been shown to be modulated by salicylic acid (SA) levels. In 2009, Du and colleagues showed that salicylic acid levels in *Arabidopsis* were controlled by release of Ca^{2+} from mitochondria in pathogenic stress conditions [61]. Therefore upon pathogenic stress, elevated ROS negatively impacts the mitochondrial membrane potential leading to an increase in cytosolic Ca^{2+} levels. This accumulation of Ca^{2+} ions in the cytosol affects

calmodulin which stimulate SA biosynthesis and in turn promotes association of TGA1 with NPR1 [59-62]. The end result is increased levels of AOX1a.

Activation pattern of mitochondrial retrograde signaling and the pathways influenced by retrograde signaling differ from species to species. For example, in yeast retrograde signaling is activated through consecutive protein-protein interactions; whereas, activation in *Arabidopsis* depends on Ca^{2+} and SA levels. In mammals, activation is dependent on release of calcium ions and interaction of calcium with different types of sensory factors. In yeast, retrograde signaling controls α-ketoglutarate/glutamate level which is important for both amino acid biosynthesis and nitrogen metabolism; whereas, in plants retrograde signaling controls ROS production as an important defense against oxidative stress and viral immunity response. In mammals, depending on the tissue type, retrograde signaling controls diverse metabolic processes including cell death, oncogenosis, development and the immune response.

Motivation

In this study, our main focus was to characterize the functional importance of Rtg2p in retrograde signaling activation. To date, studies have primarily focused on the metabolic characterization of cells under active retrograde signaling conditions. Although proteins involved in controlling retrograde signaling have been well characterized in the budding yeast *Saccharomyces cerevisiae*, little is known about the retrograde response of other fungi.

To identify retrograde signaling proteins in other yeast, the protein sequence encoded by the *Saccharomyces cerevisiae RTG2* gene was used to search for fungal homologs using NCBI BlastP. We selected four species having uncharacterized ORFs

with more than 66% amino acid identity to Rtg2p for further analysis: *Ashbya gossypii*, *Candida glabrata*, *Vanderwaltozyma polyspora*, and *Kluyveromyces lactis* (Figure 5). We set out to determine whether these genes were functional homologs of *S. cerevisiae* Rtg2p to confirm that other yeasts use a similar retrograde communication pathway between mitochondria and the nuclei. In addition, analysis of Rtg2p homologs in *rtg2*Δ of *S. cerevisiae* provides valuable insight into the functional role of Rtg2p in this pathway. Our preliminary studies showed that the Rtg2p homologs have a high degree of amino acid conservation along their length but show the greatest divergence in their C terminal domain. Using these different Rtg2p homologs has enabled us to dissect the roles of Rtg2p in retrograde signaling using naturally occuring variants of Rtg2p as functional probes.

Ashbya gossypii (*Eremothecium gossypii)* is a filamentous fungus whose natural overproduction of riboflavin makes it valuable for the industrial purposes [63]. However, *A. gossypii* is a plant pathogen that infects cotton, soybean and citrus fruits having a dramatic impact on crop loss worldwide [64]. Detailed molecular mechanisms of the signaling pathways present in this fungus are yet to be characterized.

Candida glabrata is hemiascomycetous yeast. As a human pathogen *C. glabrata* can produce candidiasis in immune-suppressed patients. After *C. albicans, C. glabrata* is the second most pathogenic yeast stain in hospital settings [65]. The increasing resistance of *C. glabrata* to azole specific antibiotics such as flucanazole makes it important organism of study. Interestingly, a mutant library screen found that increased sensitivity to azole compounds was linked to loss of proteins involved in retrograde signaling [66].

However, studies characterizing retrograde signaling *C. glabrata* have yet to be carried out.

Kluyveromyces lactis is an obligative aerobe and usually found in dairy products such as milk. Its unique metabolic activities and the overall similarity to *S. cerevisiae* make it a valuable candidate as a model organism in molecular biology and industrial applications. The presence of "DNA Killer System" in *K. lactis* is commonly used for microbial studies focusing on microbial competition. Under aerobic conditions, most yeast including *S. cerevisiae* produce ethanol from glucose leading to reduced ATP production, protein expression and biomass. However, in *K. lactis,* glycolysis is followed by the TCA cycle which allows for the production of high levels of ATP, protein expression and growth. In *S. cerevisiae,* mitochondrial retrograde signaling has an important role in synchronizing metabolic activities required for the homeostasis of the TCA cycle intermediate metabolites [67]. Despite the central role of the TCA cycle in *K. lactis,* the importance of mitochondrial retrograde regulation in *K. lactis* is unclear.

Vanderwaltozyma polyspora, also known as *Kluyveromyces polysporus*, is usually used as a model organism for research projects focused on the evolution of yeast [68]. It is the most divergent yeast strain compared *S. cerevisiae* and it has been suggested that *V. polyspora* evolved from a common ancestor which was generated by a whole genome duplication event [69].

In this study, we used the *S. cerevisiae* W303 laboratory strain as the model organism. *S. cerevisiae* have several advantages over other yeast species. First of all, our knowledge of the molecular pathways in *S. cerevisiae* is far more advanced than in other fungi. Second, the availability of molecular tools and laboratory strains gives us the

opportunity to carry out more detailed experiments. Finally, the well characterized genetics allow us to easily and quickly generate the yeast strains needed for these studies.

CHAPTER II

MATERIALS AND METHODS

Strains, growth conditions and media

The plasmids and yeast strains used in this study are presented in Tables 1 and 2, respectively. Standard yeast genetics protocols were followed to construct all strains. Strains were grown at 30^{o}C in YPD or synthetic selective media unless otherwise noted [70].

Generating *RTG2* shuffle strains

In this study, the *rtg2*Δ shuffle strain was generated as described [71]. Briefly, *RTG2* was replaced with the kanamycin marker in diploid yeast [72]. This strain was then transformed with a *URA3* marked plasmid that contained the *RTG2* gene expressed under its own promoter. The resulting diploid was sporulated and *rtg2*Δ haploid mutants containing the *URA3* covering plasmid were identified. 0.2 g/L G418 supplemented YPD plates used to identify *rtg2*Δ mutants. The resulting haploid showed wild type phenotype as deletion of the genomic copy of *RTG2* was covered by *RTG2* on a plasmid.

Additional strains were generated by crossing the *rtg2*Δ haploid shuffle strain with others generated using a similar protocol. Before experimental procedures, shuffle strains were grown on 5-FOA containing plates for 3 days at 30^{o}C to select against cells carrying the *URA3* marked *RTG2* plasmid in order to achieve *rtg2*Δ.

Plasmids and DNA manipulations

Isolation of genomic DNA (gDNA)

1 µl of fresh grown cells were suspended in 100 µl of lysis buffer (40mM Tris base, 0.5 mM EDTA pH 8.0, 0.05 % SDS, 14 mM β-ME). After 10 minutes of incubation at room temperature, tubes were incubated at 90^{o}C for 10 minutes. Lysates were cooled down to room temperature for 5 minutes and cellular debris was removed by centrifugation at 20,000x*g* for 1 minute. 1 µl of supernatant was added to a 25 µl PCR reaction as template. A detailed PCR primer list is presented in Table 3.

Subcloning of *RTG2* genes

A PCR based technique was used to generate *RTG2* genes from *S. cerevisiae*, *A. gossypii*, *C. glabrata*, *K. lactis* and *V. polyspora* using genomic DNA as a template. *K. lactis* (NRRL Y-8283) and *V. polyspora* (NRRL Y-8279) yeast cells were gifts from Dr. C. P. Kurtzman (USDA, Peoria, IL); *A. gossypii* gDNA was purchased from ATCC (Manassas, VA); *C. glabrata* (ATCC-200989) yeast cells were gift from Dr T. Edlind (Drexel University, Philadelphia, PA); *S. cerevisiae* (DGY2 -W303 strain) was from our lab. gDNA preparations were carried out from yeast strains as described above. The resulting PCR fragments were digested with specific restriction endonuclease enzymes (New England Biolabs, Ipswich, MA) and subcloned into pRS414 vector (*TRP1*, *CEN/ARS*) to generate the *S. cerevisiae RTG2* promoter *RTG2* ORF followed by a 3XHA epitope tag. All of the clones were sequenced to confirm that *RTG2* genes (1767 bp for *S. cerevisiae*; 1803 bp for *C. glabrata*; 1764 bp for *A. gossypii*; 1764 for *K. lactis*; 1803 bp

for *V. polyspora*) contained no mutations and were inframe with 3XHA epitope tag (Arizona State University). For constitutive expression based experiments, the *RTG2-3XHA* fragments were sub-cloned under *GPD* promoter in the YCplac22 plasmid (*TRP1*, 2µ). The expected molecular weights of expressed *RTG2* homologs were estimated as 65,48 kDa for *S. cerevisiae* Rtg2p (588 amino acids), 66.95 kDa for *C. glabrata* Rtg2p (600 amino acids), 64.10 kDa for *A. gossypii* Rtg2p (587 amino acids), 64.60 for *K. lactis* Rtg2p (587 amino acids) and 66.71 for *V. polyspora* Rtg2p (600 amino acids) using Serial cloner 1.3-11 software (Serial Basics, France). An addition of 3.75 kDa is also estimated for Rtg2p homologs integrated with 3XHA sequence.

Generating FLAG-Longtine epitope tagging cassette

50 µl of 20 ng/µl primer mix was prepared from Flag Sense (DG135) and Flag Antisense (DG136) oligonucleotides containing the sequence for the 3XFLAG epitope. To generate the dsDNA cassette single stranded primers were mixed together in an equal molar ratio and incubated at 95^{o}C for 20 minutes followed by cooling at room temperature for 30 minutes. The resulting product was cloned into the PacI - AscI digestion sites of pFA6a-GFP(S65T)-*HIS3MX6* (pDG59) replacing the *GFP* (S65T) fragment with the 3XFLAG cassette.

Gene deletions and integration based epitope tagging

Single gene deletions for *RTG2* and *MKS1* were carried out by a PCR based integration protocol as described [72]. Double or triple deletion/tagged strains were generated by mating of single or double deletion/tagged strains followed by tetrad dissection analysis. Tetrads were analyzed for 2:2 distribution of marker genes and

deletion of *RTG2* and *MKS1* were confirmed by genomic PCR. For protein expression experiments, *CIT2*, *MKS1* and *BMH1* were epitope tagged with 3XHA, 13XMyc and 3XFLAG epitope tags, respectively using a chromosomal integration protocol [72]. All integrations were confirmed by genomic PCR using the primers listed in Table 1.

Protein preparation

Total cellular protein samples were extracted by alkaline lysis followed by TCA precipitation as described [73]. Samples were solubilized in SDS-PAGE loading buffer via sonicatation. Unless otherwise stated, an amount of protein extract equivalent to 0.1 OD_{600} of original cell culture was used for western blot analysis.

Western blot analysis

For western blot analysis, protein samples were separated by SDS-PAGE and transferred to nitrocellulose at 14 Volts for 15 hours or 70 Volts for 3 hours at 4^{o}C. After amidoblack staining to visualize protein standards and confirm equal loading, the membranes were blocked with 1XTBS (20 mM Tris pH 7.4, 200 mM NaCl, 0.1% Tween-20) solution supplemented with either 1% BSA or 5% Carnation non-fat dry milk. Proteins were detected using primary antibodies at the dilutions listed in Table 4 and either HRP conjugated anti-mouse monoclonal or anti-rabbit polyclonal secondary antibodies. Pierce enhanced chemiluminescence detection system (Thermo Scientific, Rockford, IL) was used for detection.

Cytosolic and nuclear fractionation

Nuclear fractionations were carried out using the protocol as described by Dedon with slight modifications [74]. 10^{8} exponentially growing cells were harvested by

centrifugation and resuspended in 0.1 M Tris-SO_4 (pH 9.4), 10 mM DTT. After 15 minutes on ice, cells were treated with zymolase (1 mg zymolase/g cell pellet) for 22 minutes at 30^{o}C for spheroplast formation. Spheroplast were lysed in 1ml Spheroplast Lysis Buffer (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5, 1 mM PMSF, 1XPIM) by gentle inversion. Lysates were spun at 2,300x*g* for 5 minutes and supernatant and nuclear pellet were carefully separated. The supernatant was spun at 50,000x*g* for 30 minutes at 4^{o}C and the high speed soluble material was saved as the cytosolic fraction. Nuclear pellets were washed 3 times with ice-cold spheroplast lysis buffer before lysis in 400 µl RIPA buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with vortexing for 1 minute. The nuclear lysate was centrifuged for 30 minutes at 20,000x*g* at 4^{o}C and the resulting supernatant saved as the nuclear fraction.

Determination of doubling time

Yeast cultures were inoculated to an initial optical density reading of 0.05 at 600 nm. Spectrophotometric measurements (600 nm) were taken every 2 hours from cultures growing at 30^{o}C in a shaking incubator until the cultures reached stationary phase. Log_{10} values of each OD_{600} measurement (*log10(OD_{600})*) was plotted versus time. The linear portion of the graph (the exponential growing phase) was converted to log_2 using the formula:

$$log_2(OD600) = log10(OD_{600})/log_{10}(2)$$

The resulting log_2 values were plotted versus time and the slope of the graph used to calculate doubling time.

Analysis of codon usage profiles

Active Perl 5.10.12 was used to design a program to determine codon usage profiles for various yeasts. Data files containing genomic sequences were downloaded from NCBI (*http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi*). For each amino acid, the corresponding usage fractions were calculated and plotted. The Perl code used is presented in the Appendix A.

H_2O_2 overlay assay

$3x10^6$ cells from an exponentially growing culture were harvested by centrifugation and resuspended in 1 ml sterile water. 120 µl of the suspended cells were evenly distributed onto a -Trp selection plate using Rattler™ Plating Beads (Zymo Research, Irvine, CA). Plates were kept at room temperature for 30 minutes until the liquid had completely diffused into the media. Sterile filter papers (1.5 cm diameter circular cut) were centered on each plate and 10 µl of concentrated H_2O_2 loaded onto the center of each filter paper. After 3 days of growth at 30^oC, the radius of the zone of inhibition was measured taking 6 different radii measurements for each plate.

Protein half-life determination

Protein half-lives for Rtg2p, Mks1p, Cit2p and Pgk1p was determined using a cycloheximide chasing assay as described [75]. Briefly, 10 milliliters of 10^7 cell/ml cultures grown in –Trp selection media were collected from exponentially growing cells. Cycloheximide (Sigma-Aldrich, Saint Louis, MO) was added to a final concentration of 100 µg/ml to block cytosolic protein synthesis. Spectrophotometric measurements were taken for every 2 hours to confirm that there was no growth. 1 ml of the culture was

removed at 90 minute intervals for Rtg2p, Mks1p and Pgk1p based measurements and at 30 minutes intervals for Cit2p by centrifugation at 20,000x*g* for 1 minute. Cell pellets were frozen on dry ice and stored at -80^{o}C until processing. Protein extraction and western blot analysis were carried out as described above.

Crude mitochondria isolation

One liter cultures were grown in 2% lactate media at 30 ^{o}C in a shaking incubator until they reached an average of 1.0 OD_{600}. Cells were harvested by centrifugation and isolated as described [76].

Briefly, harvested cell pellet was resuspended in 40 ml buffer containing 0.1 M Tris-SO_4 pH 9.4 and 10 mM DTT. The suspension was incubated in a 30^{o}C water bath for 10 minutes and cells were reisolated by centrifugation at 2,800x*g* for 5 minutes at 4^{o}C. Cell pellets were resuspended in 20 mM KPO_4, 1.2 M Sorbitol (6 ml of buffer per gram of cells) and then treated with zymolase (1mg/gram of cells) until 90% of the cells were converted to spheroplasts. Spheroplasts were washed in 1.2 M sorbitol three times followed by centrifugation (2,800x*g* for 5 minutes at 4^{o}C). After resuspension of spheroplasts in 40 ml of Buffer A (40 mM Hepes, pH 7.5, 1.2 M Sorbitol, 20U/ml Trasylol, 2 mM p-aminobenzamidine, 1 mM EDTA, 1 mM PMSF and 10 mM DTT), homogenization was carried out by douncing ~20 times using a tight fitting piston in a 40 ml dounce. Homogenates were spun at 1,700x*g* for 4 minutes at 4^{o}C to remove nuclear and unbroken cells. The pellet was rehomogenized as described above while supernatant was kept on ice. Secondary supernatant was pooled with the first and spun at 1,700x*g* for 4 minutes at 4^{o}C. The mitochondria were pelleted at 10,000x*g* for 10 minutes at 4^{o}C. After aspiration, the mitochondrial pellet was gently resuspended in 1XBuffer A without

EDTA or DTT but with 2 mM citrate. The protein concentration was determined at OD_{280} by mixing 1 ml of 0.6% SDS with 10 µl mitochondria. An OD_{280} reading of 0.2 is equivalent to 10 mg/ml protein. Mitochondrial aliquots were prepared at 0.5 mg mitochondria/tube and stored at -80^{o}C by adding equal volumes of freezing buffer (20 mM Hepes pH 7.4, 0.6 M Sorbitol, 20 mg/ml BSA, 20% DMSO, 1mM PMSF, 50 U/ml Trasylol).

In-gel aconitase assay

Aconitase activity was measured using an in-gel assay as previously described [77]. Briefly, 200 ng isolated mitochondria were washed with isotonic buffer (Tris-HCl pH 8.0, 0.6 M Sorbitol, 2 mM Na-Citrate, and 1 mM PMSF) and resuspended in 50 µl lysis buffer (20 mM Tris-HCl pH 8.0, 50 mM NaCl, 1% Triton X-100, 10% Glycerol, 2 mM Na-Citrate, 10U Catalase, 1 mM PMSF, 1XPIM). Samples were incubated on ice for 30 minutes before loading the gel. Mitochondrial lysates were separated on a 6% acrylamide separating gel composed of 132mM Tris base, 132mM boric acid and 3.6 mM citric acid. Separation was carried out by running the gel overnight at 70 Volts and 4ºC in electrophoresis buffer containing 25 mM Tris pH 8.3, 192 mM glycine, and 3.6 mM citrate. Aconitase activity was detected by incubating the gel in activation buffer (100 mM Tris pH 8.0, 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM $MgCl_2$, 1.2 mM (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase) in the dark at 37^{o}C for 20 minutes with shaking. The reaction was stopped by washing the gel in double distilled water three times at 10 minute intervals.

Co-immunoprecipitation

$2x10^7$ cells were harvested from exponentially growing cultures by centrifugation and resuspended in 0.1 M Tris-SO_4 pH 9.4, 10 mM DTT. After 15 minutes on ice, cells were treated with zymolase (1 mg zymolase/g cell pellet) for 25 minutes at 30°C for spheroplast formation. Spheroplast were lysed in 225 µl RIPA buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with half volume of acid washed glass beads by 30 second vortexing followed by 45 second incubation on ice (cycle repeated 5 times). Total cell extracts were then spun at 20,000x*g* for 15 minutes at 4°C. 100 µl of supernatant was transferred to a new tube containing 25 µl of pre-washed EzviewTM Red Anti-c-Myc or Anti-c-Flag Affinity Gels (Sigma-Aldrich, Saint Louis, MO). 10 µl of remaining supernatant (10% of total) was saved as *Load*. The lysates were incubated with affinity gel matrix at 4°C for 2 hours on a 3-dimensional rocker. After incubation, the matrix was pelleted at 2,300x*g* for 3 minutes at 4°C and 12.5 µl of supernatant saved as *Unbound* (10 % of total). The affinity beads were washed in RIPA buffer 3 times with 15 minutes incubation periods at 4°C with rocking. Residual RIPA buffer was removed by aspiration and the bound proteins released with the addition of 50 µl of SDS Loading Buffer and saved as *Bound* (87.5% of total). Samples were stored at -80°C until processing by SDS-PAGE and western blot analysis.

Spotting assay

10^7 cells were harvested from exponentially growing cultures. To remove residual media, cells were resuspended in sterile dH_2O and repelleted by centrifugation at 12,000x*g* for 1 minute at room temperature. Pelleted cells were resuspended in 100 µl of

sterile distilled dH_2O. Cell suspensions were transferred to 96-well plates. Dilutions were carried out by serial transferring 10 µl cell suspension to successive wells containing 90 µl sterile dH_2O. 2 µl of each dilution was spotted on selective plates. Plates were incubated at 30^oC for 3 days before scoring growth.

Table 1. Plasmids used in this study

Plasmid Name	Description	Reference
pDG54	pFA6a-*KanMX6*	[72]
pDG62	pFA6a-3XHA-*HIS3MX6*	[72]
pDG59	pFA6a-GFP(S65T)-*HIS3MX6*	[72]
pDG264	pFA6a-3XFLAG-*HIS3MX6*	This Study
pDG49	YCplac22-$GPD_{promoter}$ 2µ, Amp^r, *TRP1*	[78]
pDG97	pRS414 *CEN/ARS*, Amp^r, *TRP1*	[79]
pDG98	pRS416 *CEN/ARS*, Amp^r, *URA3*	[79]
pDG127	PRS416-$RTG2_{promoter}$ *RTG2*, *CEN/ARS*, Amp^r, *URA3*	This Study
pDG176	YCplac22-$GPD_{promoter}$ $RTG2_{S.\ cerevisiae}$, 2µ, Amp^r, *TRP1*	This Study
pDG177	YCplac22-$GPD_{promoter}$ $RTG2_{C.\ glabrata}$, 2µ, Amp^r, *TRP1*	This Study
pDG178	YCplac22-$GPD_{promoter}$ $RTG2_{V.\ polyspora}$, 2µ, Amp^r, *TRP1*	This Study
pDG184	YCplac22-$GPD_{promoter}$ $RTG2_{K.\ lactis}$, 2µ, Amp^r, *TRP1*	This Study
pDG194	YCplac22-$GPD_{promoter}$ $RTG2_{A.\ gossypii}$, 2µ, Amp^r, *TRP1*	This Study
pDG193	pRS414 –$RTG2_{promoter}$ *Cen/Ars*, Amp^r, *TRP1*	This Study
pDG198	pRS414-$RTG2_{promoter}$ $RTG2_{S.\ cerevisiae}$, *CEN/ARS*, Amp^r, *TRP1*	This Study
pDG199	pRS414-$RTG2_{promoter}$ $RTG2_{C.\ glabrata}$, *CEN/ARS*, Amp^r, *TRP1*	This Study
pDG196	pRS414-$RTG2_{promoter}$ $RTG2_{K.\ lactis}$, *CEN/ARS*, Amp^r, *TRP1*	This Study
pDG197	pRS414-$RTG2_{promoter}$ $RTG2_{V.\ polyspora}$, *CEN/ARS*, Amp^r, *TRP1*	This Study

Table 1.(Continued)

Plasmid Name	Description	Reference
pDG231	YCplac22-*$GPD_{promoter}$ $RTG2_{S.\ cerevisiae}$-3XHA*, 2μ, *Amp^r*, *TRP1*	This Study
pDG221	YCplac22-*$GPD_{promoter}$ $RTG2_{C.\ glabrata}$-3XHA*, 2μ, *Amp^r*, *TRP1*	This Study
pDG209	YCplac22-*$GPD_{promoter}$ $RTG2_{A.\ gossypii}$-3XHA*, 2μ, *Amp^r*, *TRP1*	This Study
pDG248	YCplac22-*$GPD_{promoter}$ $RTG2_{K.\ lactis}$-3XHA*, 2μ, *Amp^r*, *TRP1*	This Study
pDG210	YCplac22-*$GPD_{promoter}$ $RTG2_{V.\ polyspora}$-3XHA*, 2μ, *Amp^r*, *TRP1*	This Study
pDG246	pRS414-*$RTG2_{promoter}$ $RTG2_{S.\ cerevisiae}$-3XHA, CEN/ARS, Amp^r, TRP1*	This Study
pDG229	pRS414-*$RTG2_{promoter}$ $RTG2_{C.\ glabrata}$-3XHA, CEN/ARS, Amp^r, TRP1*	This Study
pDG232	pRS414-*$RTG2_{promoter}$ $RTG2_{A.\ gossypii}$-3XHA, CEN/ARS, Amp^r, TRP1*	This Study
pDG250	pRS414-*$RTG2_{promoter}$ $RTG2_{K.\ lactis}$-3XHA, CEN/ARS, Amp^r, TRP1*	This Study
pDG227	pRS414-*$RTG2_{promoter}$ $RTG2_{V.\ polyspora}$-3XHA, CEN/ARS, Amp^r, TRP1*	This Study

Table 2. Strains and plasmids constructed in this study

Strain		Genotype*	Parent
DGY4**	a/α	*leu2-3,12/- trp1-1/- can1-100/- ura3-1/- ade2-1/- his3-11,15/-*	WT (W303)
DGY54	a/α	*rtg2*Δ::KAN^r */RTG2*	DGY4
DGY59	a	*rtg2*Δ::KAN^r	DGY54
DGY60	α	*rtg2*Δ::KAN^r	DGY54
DGY161	a	*CIT2-3XHA::HIS3*	DGY58
DGY159	α	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r*	DGY58 x DGY59
DGY160	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r*	DGY58 x DGY59
DGY138	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG97	DGY160
DGY138	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG198	DGY160
DGY140	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG199	DGY160
DGY141	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG195	DGY160
DGY142	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG196	DGY160
DGY143	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG197	DGY160
DGY122	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG49	DGY160
DGY123	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG176	DGY160
DGY124	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG177	DGY160
DGY126	a	*CIT2-3XHA::HIS3 rtg2*Δ*::KAN^r* pDG184	DGY160

Table 2.(Continued)

Strain		Genotype*	Parent
DGY127	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG178	DGY160
DGY164	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG246	DGY160
DGY153	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG229	DGY160
DGY165	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG232	DGY160
DGY177	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG250	DGY160
DGY154	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG227	DGY160
DGY149	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG231	DGY160
DGY150	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG221	DGY160
DGY151	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG209	DGY160
DGY176	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG248	DGY160
DGY152	a	*CIT2-3XHA::HIS3 rtg2Δ::KAN^r* pDG210	DGY160
DGY166	α	*MKS1-13XMyc::HIS3*	DGY58
DGY175	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r*	DGY160 x DGY166
DGY178	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG97	DGY175
DGY179	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG231	DGY175
DGY180	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG229	DGY175
DGY181	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG209	DGY175

Table 2.(Continued)

Strain		Genotype*	Parent
DGY182	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG232	DGY175
DGY183	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG250	DGY175
DGY184	a	*MKS1-13XMyc::HIS3 rtg2Δ::KAN^r* pDG227	DGY175
DGY206	α	*mks1Δ::KAN^r*	DGY58
DGY211	α	*mks1Δ::KAN^r rtg2Δ::KAN^r*	DGY60 x DGY206
DGY212	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r*	DGY60 x DGY206
DGY216	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG97	DGY212
DGY217	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG231	DGY212
DGY218	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG229	DGY212
DGY219	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG232	DGY212
DGY220	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG209	DGY212
DGY221	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG250	DGY212
DGY222	a	*CIT2-3XHA::HIS3 mks1Δ::KAN^r rtg2Δ::KAN^r* pDG227	DGY212
DGY239	α	*BMH1-3XFLAG::HIS3*	DGY58
DGY231	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2Δ::KAN^r*	DGY175 x DGY239
DGY240	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2Δ::KAN^r* pDG97	DGY231
DGY241	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2Δ::KAN^r* pDG246	DGY231

Table 2.(Continued)

Strain		Genotype*	Parent
DGY242	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2*Δ*::KAN*^{r} pDG229	DGY231
DGY243	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2*Δ*::KAN*^{r} pDG209	DGY231
DGY244	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2*Δ*::KAN*^{r} pDG250	DGY231
DGY245	a	*MKS1-13XMyc::HIS3 BMH1-3XFLAG::HIS3 rtg2*Δ*::KAN*^{r} pDG227	DGY231

* All strains are derivatives of W303 and are *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15* pDG127 unless otherwise noted. Strains carrying yeast genes on plasmids are indicated.

** Wild type strain does not carry pDG127 plasmid.

Table 3. PCR primers used in this study

Primer	Description	Sequence
DG13	*RTG2* deletion, Longtine F1	5'CTAAGGATTGTTTTGAACGAAAAGTGTAGGCGTGCCACAACG GATCCCCGGGTTAATTAA3'
DG14	*RTG2* deletion, Longtine R1	5'GGATTTCGTATTTATTGTTCAAGTATTTAAAGACTAGATGTGA ATTCGAGCTCGTTTAAAC3'
DG15	*RTG2* promoter, Sense	5'CGCGAAGCTTCTAATGGCAATTACGAGTGTTTTCATAGC3'
DG16	*RTG2* ORF, Antisense	5'CGCGAGATCTCTCGAGTTCTTCATAAAATTGCACGCC3'
DG17	Kanamycin deletion test, Sense	5'GGCTCCTCGCTGCAGACCTGCGAGC3'
DG18	Kanamycin deletion test, Antisense	5'GCTCGCAGGTCTGCAGCGAGGAGCCG3'
DG34	*CIT2* 3XHA tagging, Longtine F1	5'CTGAGAAATACAAGGAATTGGTCAAAAACATTGAAAGCAAA CTACGGATCCCCGGGTTAATTAA3'
DG35	*CIT2* 3XHA tagging, Longtine R1	5'CGAGGAAGGAAATAGTAACGTTCATTTTCTTAATTATAAATA TCGAATTCGAGCTCGTTTAAAC3'
DG42	*CIT2* tagging verification, Sense	5'CTTGCTCAATTGATCACTGATAGG3'
DG43	*HIS5* test, Antisense	5'CGATACATTCAACAATAAGAGACCA3'
DG48	*RTG2* ORF (*S. cerevisiae*), Sense	5'GCGCCTCGAGATGTCAACACTTAGCGATAGTGATACC3'
DG49	*RTG2* ORF (*S. cerevisiae*), Antisense	5'GCGCCTCGAGTTAAGATCTTTCTTCATAAAATTGCACGCCAAT TTTAACCC3'
DG51	*RTG2* ORF (*K. lactis*), Antisense	5'GCGCCTCGAGTTAGGATCCATAGAATTCTACATTGATCTTAAC CTTGTC3'

Table 3.(Continued)

Primer	Description	Sequence
DG52	*RTG2* ORF (*C. glabrata*), Sense	5’GCGCCTCGAGATGTCGGTATTATCTGATAGCGATACTG3’
DG53	*RTG2* ORF (*C. glabrata*), Antisense	5’GCGCCTCGAGTTAAGATCTATTATCTGAGAATTGTACAGATATCCGTACTTTTTC3’
DG54	*RTG2* ORF (*A. gossypii*), Sense	5’GCGCCTCGAGATGTCGGCTATTTCAGATAGCGATACAG3’
DG55	*RTG2* ORF *(A. gossypii*), Antisense	5’GCGCCTCGAGTTAAGATCTGCACGCCTCTGAAAACTGTACCCCTACCTTGACC3’
DG56	*RTG2* ORF (*V. polyspora*), Sense	5’GCGCCTCGAGATGTCTGCGATATTGGACATTACTGA3’
DG57	*RTG2* ORF (*V. polyspora*), Antisense	5’GCGCCTCGAGTTAAGATCTTGATTGAGTTTGCTGCTCTTCAGTGAATTGGACAC3’
DG66	*RTG2* promoter, Sense	5’GCGCCCCGGGGCTAGCCTGCAGCAGTTATTCACCCGGAGGCACAC3’
DG67	*RTG2* promoter, Antisense	5’GCGCCTCGAGTTGTGGCACGCCTACACTTTTCGTTC3’
DG68	*RTG2* ORF (*S. cerevisiae*), Sense	5’GCGCGGTACCTTAAGATCTTTCTTCATAAAATTGCACGCCAATTTTAACCCTC3’
DG70	*RTG2* ORF (*C. glabrata*), Antisense	5’GCGCGGTACCTTAAGATCTATTATCTGAGAATTGTACAGATATCCGTACTTTTTC3’
DG71	*RTG2* ORF (*A. gossypii*), Antisense	5’GCGCGGTACCTTAAGATCTGCACGCCTCTGAAAACTGTACCCCTACCTTGACC3’
DG93	*MKS1* deletion, Longtine F1	5’ATGTCGCGGGAGGCATTTGATGTACCGAATATAGGTACTCGGATCCCCGGGTTAATTAA3’

Table 3.(Continued)

Primer	Description	Sequence
DG94	*MKS1* deletion, Longtine R1	5’CATTATCTTTTTTTGAAAGAACTTTAAATACTGTATCTG GAATTCGAGCTCGTTTAAAC3’
DG124	*MKS1* deletion detection, Antisense	5’CAATGGCTTCCCCTGAGCTAGCGC3’
DG98	*MKS1* 13XMyc tagging, Longtine F2	5’GAAGCACTGGGGCGTAAGACGAGTAATGGAGGGCGAATACG GATCCCCGGGTTAATTAA3’
DG99	*MKS1* 13XMyctagging, Longtine R1	5’CATTATCTTTTTTTGAAAGAACTTTAAATACTGTATCTG GAATTCGAGCTCGTTTAAAC3’
DG103	*MKS1* tagging detection, Sense	5’ATGGTACGTACAATATCACGG3’
DG121	*BMH1* 3XFLAG tagging, Longtine F2	5’CAACAGCAGCCACCTGCTGCCGCCGAAGGTGAAGCACCAAA GCGGATCCCCGGGTTAATTAA3’
DG122	*BMH1* 3XFLAG tagging, Longtine F2	5’TATAGATATATAATTGCAATAATGAACTACAAATTATTACAC GAATTCGAGCTCGTTTAAAC3’
DG123	*BMH1* tagging verification, Sense	5’CTTATCATGCAACTGCTAAGG3’
DG134	pDG59 3XFLAG cloning, Sense	5’TAACGATTATAAAGATGACGATGACAAGGATTATAAAGATGA CGATGACAAGGATTATAAAGATGACGATGACAAGTAAGG3’
DG135	pDG59 3XFLAG cloning, Antisense	5’CGCGCCTTACTTGTCATCGTCATCTTTATAATCCTTGTCATCGT CATCTTTATAATCCTTGTCATCGTCATCTTTATAATCGTTAAT3’

Table 4. Antibodies used for western blot analysis

Target	Epitope	Antibody	Dilution	Company/Lab-Clone #	Origin of Antibody	Blocking Solution (in 1XTBS + 0.1% Tween20)
Rtg2p Homologs	*HA*	Anti-HA	1:5,000	Covance-16B12	Mouse-Monoclonal	1% BSA
Cit2p	*HA*	Anti-HA	1:5,000	Covance-16B12	Mouse-Monoclonal	1% BSA
Mks1p	*Myc*	Anti-Myc	1:5,000	Covance-9E10	Mouse-Monoclonal	1% BSA
Bmh1p	FLAG	Anti-FLAG	1:5,000	Covance-PBR-132C	Mouse-Monoclonal	1% BSA
Aco1p	Native	Anti-Aco1	1:10,000	Gift from A. Dancis, UPENN-218	Rabbit-Polyclonal	5% Milk
Pgk1p	Native	Anti-Pgk1	1:5,000	Invitrogen-22C5	Mouse-Monoclonal	5% Milk
Tom40p	Native	Anti-Tom40	1:10,000	Gift from D. Pain UMDNJ	Rabbit-Polyclonal	5% Milk
Act1p	Native	Anti-Actin	1:3,000	Abcam-ab40864	Mouse-Monoclonal	5% Milk
Histone H2B	Native	Anti-H2B	1:3000	Active Motif-03108001	Rabbit-Polyclonal	5% Milk

CHAPTER III

RESULTS

RTG2 complementation of *rtg2*Δ phenotypes

Studies have shown that cells deleted for *RTG2* are glutamate auxotrophs, have reduced aconitase activity, and decreased levels of Cit2p. The glutamate auxotrophy of *rtg2*Δ illustrates the central function of Rtg2p in multiple biological pathways including nitrogen metabolism, the synchronization of the TCA cycle with the glyoxylate cycle, and amino acid biosynthesis.

To determine whether expression of fungal Rtg2p homologs had an impact on the growth of *rtg2*Δ, the doubling time for each strain was calculated. We found that expression of Rtg2p homologs had no negative effect on growth under normal conditions and on average all strains had a doubling time of 93 minutes (Figure 6). To determine whether the *RTG2* genes coded for functional homologs of *S. cerevisiae* Rtg2p, their ability to complement *rtg2*Δ glutamate auxotrophy was tested using our *rtg2*Δ shuffle stain. Note that in our hands, cells deleted for *RTG2* are auxotrophic for glutamate, but not proline as described by Velot. (Figure 7) [15]. This discrepancy may be due to the differences in the strains used (W303, this study; BY4741, Velot et al.). When expressed under the endogenous *RTG2* promoter, all Rtg2p homologs complemented growth on media lacking glutamate except for Rtg2p from *A. gossypii* (Figure 8-A). To determine whether this lack of complementation was due to expression level differences, all *RTG2*

homologs were placed under the strong, constitutive *GPD* promoter and retested for glutamate auxotrophy. Under these conditions, all Rtg2p homologs complemented growth on media lacking glutamate suggesting that all homologs are functional in *S. cerevisiae* and the lack of complementation by *A. gossypii* was related to reduced levels of Rtg2p (Figure 8-B).

Measuring the sensitivity of *rtg2*Δ mutants to different stress conditions

Under laboratory conditions, yeast cells can be propagated under a wide range of environmental conditions including variations in carbon sources, nitrogen sources, temperatures, etc. Survival therefore depends on their ability to adapt their metabolism to these different conditions. A simple eukaryotic organism, yeast have evolved complex survival strategies that involve overlapping signal transduction pathways. To determine whether retrograde signaling had a role in cellular response to different stress conditions, we compared the growth of our strains under heavy metal, salt, high/low temperature, varying carbon source, cytotoxic agent and osmotic stress conditions. We identified no essential requirement for Rtg2p under these conditions as all strains grew to the same extent suggesting that Rtg2p is not involved in the general adaptive stress response pathway (Figure 9). However, *rtg2*Δ mutants grown in the presence of 7 mM caffeine did show slower growth when compared to WT cells (Figure 9-F). Since caffeine is a cytotoxic agent that inhibits DNA repair mechanisms in cells [78], these results suggest that there may be a link between retrograde signaling and DNA repair mechanisms although the exact role remains to be tested. Interestingly, *rtg2*Δ mutant cells showed

significantly increased sensitivity to oxidative stress introduced by H_2O_2 indicating that Rtg2p has an important role in oxidative stress response (Figure 10-A). To determine whether the Rtg2 homologs also function in a similar manner, an overlay assay was carried out using strains expressing *RTG2* genes under both the native *RTG2* promoter and the constitutively active *GPD* promoter.

Cells deleted for *RTG2* are 35-45% more sensitive to H_2O_2 treatment and expression of all *RTG2* homologs rescued this sensitivity (Figure 10-B). Interestingly, constitutive expression of *RTG2* genes did not result in increased resistance to H_2O_2 (Figure 10-C) suggesting the role of Rtg2p in the resistance to externally added H_2O_2 is not controlled by the expression level of Rtg2p. Interestingly, we found that WT cells were more sensitive to H_2O_2 than *rtg2*Δ cells when retrograde signaling activation was induced by removal of glutamate from the media (Figure 11). These results suggest that the sensitivity to H_2O_2 may be a result of indirect effects related to cellular activities responsive to retrograde signaling activity.

Half-lives of Rtg2p homologs and Mks1p

Studies by Liu and colleagues have shown that Mks1p levels are controlled by ubiquitin dependent degradation via Grr1p, the F box component of the SCF [47]. Mks1p not bound to either Rtg2p or Bmh1p is subjected to polyubiquitination and degradation by the 26S proteosome [47]. In our studies, *rtg2*Δ mutant cells expressing *A. gossypii* Rtg2p showed significantly reduced levels of Rtg2p when compared to other strains (Figure 12). Given the link between Rtg2p-Mks1p interaction and the finding that

unbound Mks1p is a target for degradation, we set out to determine whether the loss of Rtg2p had an impact on the stability of Mks1p. We first measured the half-life of Rtg2p using a cycloheximide based pulse-chase protocol collecting samples at 1.5 hr intervals over a 6 hour period. In a previous study, Belle calculated a half-life of ~30 minutes for Cit2p and greater than 6-hour half-life for Pgk1p, a finding that was repeatable in our hands [75]. In contrast, we calculated ~3-hour half-life for Rtg2p which was much greater than the 70 minutes determined by Belle. In their study, measurements were taken for only 45 minutes and half-life calculated using a mathematical model. Our data was collected over a longer period of time and may therefore better represent the actual half-life for Rtg2p

From our results we conclude that, the reduced Rtg2 levels for *A. gossypii* was not due to decreased polypeptide stability as the calculated half-life for *A. gossypii* Rtg2p was similar to that for *S. cerevisiae* Rtg2p (Figure 13-A and Table 5). The calculated half-life for *C. glabrata, K. lactis* and *V. polyspora* Rtg2p proteins was greater than that for *S. cerevisiae* Rtg2p. Interestingly, this increased stability for Rtg2p was paralleled by an increased stability for Mks1p in these strains.

Analysis of Cit2p and Aco1p expression levels

Retrograde signaling in yeast integrates the glyoxylate cycle with the demands of cellular metabolism. Cit2p and Aco1p are two key enzymes involved in the glyoxylate cycle. *CIT2* mRNA levels have been shown to increase with the activation of the retrograde signaling pathway [8, 49], while Velot and colleagues found a 50% reduction

in Aco1p activity in cells deleted for *RTG2* [15]. We therefore tested expression of both Cit2p and Aco1p in our WT and *rtg2*Δ strains. We found that deletion of *RTG2* completely abolished Cit2p protein levels and reduced the expression of Aco1p by 50% when compared to a WT strain (Figure 14-A). Expression of *RTG2* homologs in the *rtg2*Δ shuffle strain reverted the levels of Cit2p and Aco1p to WT levels for *C. glabrata* and *K. lactis.* However, expression of the Rtg2p homolog from *A. gossypii* or *V. polyspora* in *rtg2*Δ mutant was unable to rescue Cit2p or Aco1p expression. To determine whether overexpression of *RTG2* could compensate for this apparent loss of activation, we measured Cit2p and Aco1p levels in strains expressing Rtg2p homologs from the *GPD* promoter. We found that elevated expression of *A. gossypii* and *V. polyspora RTG2* genes increased Cit2p levels to 50% of WT (Figure 14-B) but had no effect on Aco1p expression. The increased protein level of Cit2p for Rtg2p homologs from *A. gossypii and V. polyspora* expressed from constitutive *GPD* promoter suggests that the expression level of Rtg2p might be a regulatory factor for the retrograde signaling pathway. To determine whether this may be the case, we measured the levels of Rtg2p homologs in the presence and absence of glutamate. Results showed that Rtg2p levels did not vary in response to changes in the availability of glutamate (Figure 15) suggesting that activation of Rtg2p, if regulated, may be at a post-translational level.

Aco1p contains a 4Fe-4S cluster that is susceptible to damage. To determine whether the Aco1p activity paralleled the steady state levels of Aco1p, we isolated mitochondria from our *rtg2*Δ shuffle mutants expressing Rtg2p homologs and measured Aco1p activity using an in gel assay as described [77]. Similar to the reduction in Aco1p

levels, Aco1p activity was also reduced in *rtg2*Δ and *rtg2*Δ cells expressing the *A. gossypii* and *V. polyspora* Rtg2p homologs (Figure 16).

We also asked whether the reduction in Aco1p and Cit2p can be bypassed by deletion of *MKS1*, the negative regulator of retrograde signaling. Western blot data showed that by deleting *MKS1* in the *rtg2*Δ background Aco1p and Cit2p expression returned to WT levels. All of our *rtg2*Δ that showed reduced Aco1p and Cit2p levels had WT level expression for both Aco1p and Cit2p in the *msk1*Δ*/rtg2*Δ double mutant (Figure 17). As stated in the literature, cells deleted for *MKS1* constitutively activate the retrograde signaling pathway regardless of Rtg2p status [80]. Similarly, we also found the expression of Cit2p was increased 3-4 fold upon deletion of *MKS1* or absence of glutamate (Figures 17). This suggests that retrograde signaling is completely independent of Rtg2p function upon deletion of *MKS1*. On the other hand, *rtg2*Δ cells expressing Mks1p and different Rtg2p homologs showed different profiles depending on the availability of glutamate (Figure 18). Note that we were not able to collect samples from either *rtg2*Δ or *rtg2*Δ expressing Rtg2p from *A. gossypii* under inducing conditions as these strains are unable to grow in the absence of glutamate. We did find that the expression level of Cit2p was increased in cells expressing *S. cerevisiae*, *C. glabrata* and *K. lactis* Rtg2p homologs when they were grown in media lacking glutamate; however, Cit2p expression was still only 20-30% of WT level for Rtg2p from *V. polyspora* Rtg2p (Figure 18). Given that *rtg2*Δ cells expressing *V. polyspora* Rtg2p were able to rescue cells from glutamate auxotrophy, the function of Rtg2p is likely sufficient to complement glutamate auxotrophy yet not sufficient for complete *CIT2* transcriptional activation.

The expression profile for Aco1p was unique compared to that of Cit2p. We detected a minor increase in Aco1p expression in cells grown in the absence of glutamate for all Rtg2p homologs which increased expression by 2-3 fold upon deletion of *MKS1* (Figure 18). Data shows Rtg2p influences the expression of Aco1p, yet expression is not completely dependent on Rtg2p function.

Studies have shown that TOR functions as an inhibitor of the retrograde signaling pathway [29]. We therefore measured expression of Cit2p and Aco1p in cells treated with rapamycin, a TORC1 inhibitor. In WT cells grown in the presence of glutamate, the rapamycin treatment induces a 4 fold increase in Cit2p expression which is twice the Cit2p levels found in cells grown in the absence of glutamate (Figure 19). Interestingly, the rapamycin dependent induction is lost when *MKS1* is deleted returning the expression level of Cit2p to that observed in the absence of glutamate. In cells deleted only for *rtg2*Δ, rapamycin also failed to induce Cit2p expression suggesting that rapamycin dependent induction of Cit2p expression requires both Rtg2p and Mks1p.

We also analyzed the expression profile of Aco1p in the absence of glutamate but with rapamycin treatment. Results for Aco1p expression profiles are presented in Figures 17 and 18. In general, changes in Aco1p levels followed a similar pattern as that seen for Cit2p for both *rtg2*Δ single and *rtg2*Δ*mks1*Δ double mutants. As described previously, deletion of *MKS1* results in increased Aco1p expression [81]. In the absence of glutamate, cells expressing Rtg2p homologs showed a minor increase in Aco1p levels suggesting that Cit2p and Aco1p expression are controlled simultaneously under these conditions. However, in contrast to increased Cit2p levels, WT Aco1p levels were

partially reduced under rapamycin treatment suggesting that the expression of Cit2p and Aco1p is likely independently controlled under rapamycin treatment.

As expected, expression profiles for Cit2p and Aco1p in *rtg2*Δ and *rtg2*Δ*mks1*Δ strains expressing the *A. gossypii* Rtg2p homolog were similar to expression profiles for *rtg2*Δ and *rtg2*Δ*mks1*Δ mutants.

In summary, the induced expression profiles for both Cit2p and Aco1p require expression of a functional Rtg2p in the absence glutamate. However, in the presence of rapamycin, elevated expression of Cit2p requires both functional Rtg2p and Mks1p. The fact that rapamycin treatment appears to negatively regulate expression of Aco1p, even in the presence of functional Rtg2p and Mks1p, suggests that Aco1p expression is under the control of overlapping pathways that modulate expression based on different conditions.

Analysis of Mks1p-Rtg2p interaction

Control of retrograde signaling involves many different proteins. Mks1p is a known negative regulator of the pathway and it is believed that Mks1p, along with Bmh1p, inhibits the pathway by preventing nuclear localization of Rtg1p/Rtg3p. Because of the involvement of Mks1p in retrograde signaling, we measured the ability of Mks1p to interact with Rtg2p homologs in our *rtg2*Δ shuffle strain. Compared to Rtg2p from *S. cerevisiae*, Rtg2p homologs from *C. glabrata, K. lactis* and *V. polyspora* showed 50-80% lower affinity for Mks1p binding (Figure 19 and Table 8). Similar to that reported for Rtg2p [82], all Rtg2p homologs showed an increased association with Mks1p (~2 fold) when retrograde signaling was induced. Interestingly, *V. polyspora* Rtg2p homolog

showed a similar binding affinity for Mks1p as other homologs. However given that *V. polyspora* was unable to complement Cit2p expression together these results suggest that interaction between Rtg2p and Mks1p is alone insufficient to transmit a retrograde response. In our studies, we also found a free pool of Rtg2p (~80-90% of total) and Mks1p (~70-80% of total) for which a function has yet to be determined.

Analysis of Mks1p-Bmh1p interaction

In addition to Mks1p, Bmh1p also functions as a negative regulator of the retrograde signaling pathway. The dynamic interaction between Mks1p and Bmh1p is the current model for retrograde signaling activation. The decreased association between Mks1p and Bmh1p results in increased association of Mks1p with Rtg2p [46]. Our data for Mks1p-Rtg2p interaction showed a reduced interaction between Mks1p and Rtg2p homologs compared to WT. We therefore analyzed the interaction between Mks1p and Bmh1p to determine whether Rtg2p homologs had any effect on this interaction. In the presence of glutamate, Mks1p showed similar binding affinity for Bmh1p irrespective of the expressed Rtg2p homolog (Figure 20 and Table 9). Interaction between Bmh1p and Mks1p has been shown to be reduced in cells grown in the absence of glutamate [46]. Interestingly, Mks1p-Bmh1p interaction was stronger in *rtg2*Δ cells expressing *S. cerevisiae* Rtg2p when compared to other Rtg2p homologs, and was significantly reduced in the absence of glutamate. This reduction in affinity was not as great as that seen for fungal Rtg2p homologs. These results suggest a functional role for Rtg2p in Mks1p interaction that affects the downstream dynamic interaction of Mks1p with Bmh1p.

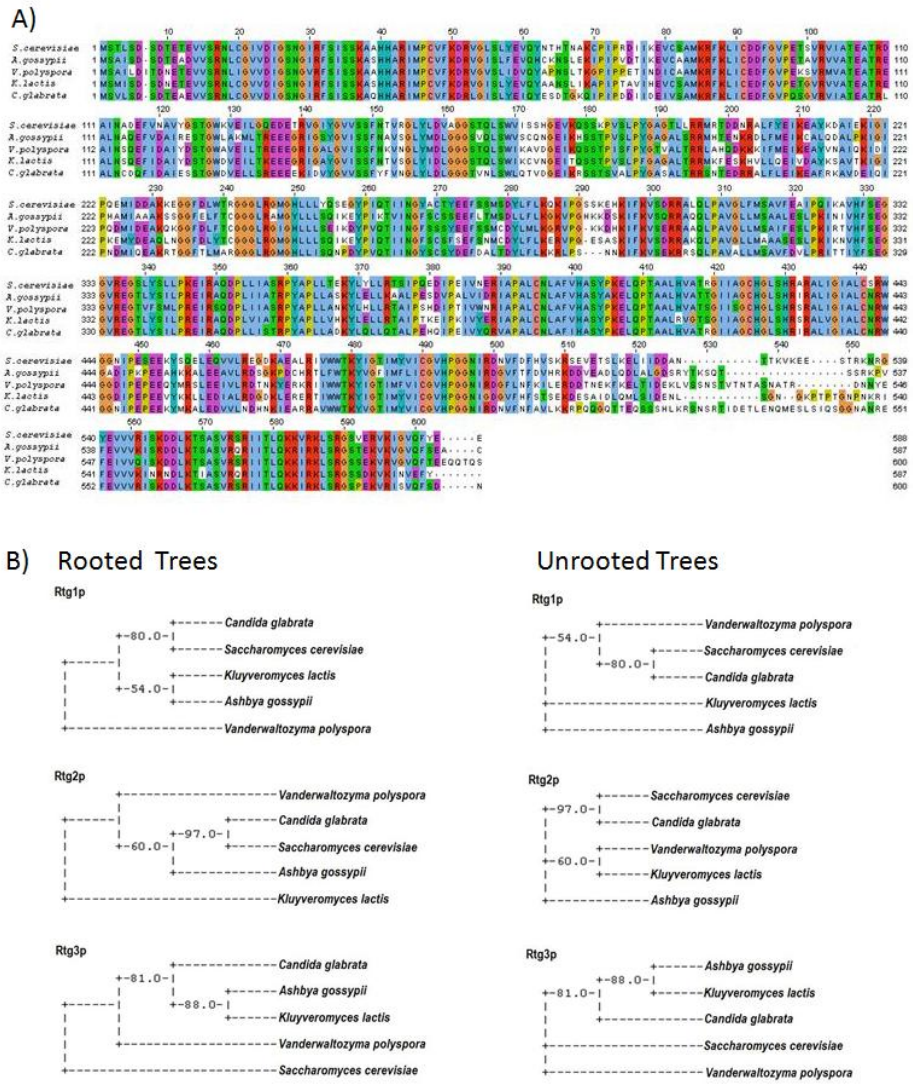
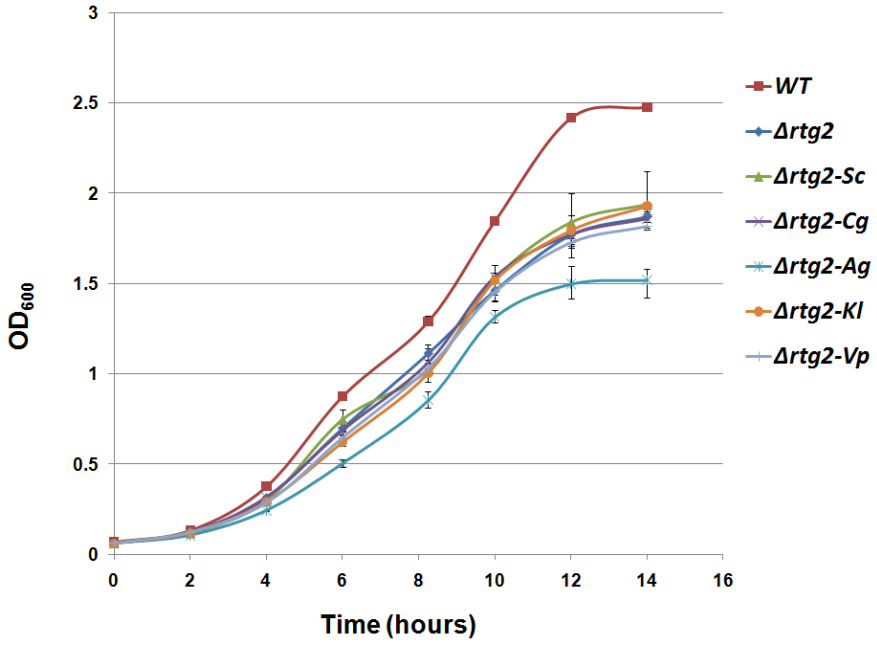


Figure 5. Multiple sequence alignments for Rtg2p

A) Multiple sequence alignments generated using the T-Coffee Alignment Tool [83]

B) Distance output data of multiple sequence alignment analysis were used for construction of Rooted and Unrooted Trees by consecutive operation of the Seqboot, Protdist, Neighbour, and Consense program of the PHYLIP 3.68 phylogenetic inference package [84]



Strain	Doubling Time, n = 3 (minutes)
WT	86 ± 0.04
Δrtg2	96 ± 0.06
Δrtg2-Sc	91 ± 0.06
Δrtg2-Cg	93 ± 0.06
Δrtg2-Ag	100 ± 0.12
Δrtg2-Kl	95 ± 0.10
Δrtg2-Vp	95 ± 0.08

Figure 6. Doubling times for strains expressing Rtg2p homologs in *S. cerevisiae*

Spectrophotometric measurements at 600 nm were taken at 2 hour intervals for wild type (WT) and *rtg2*Δ shuffle strains grown in selective media at 30°C with shaking. The change in OD_{600} over time is shown for all Rtg2p homologs (top panel). Doubling times (bottom panel) are calculated as described in *Materials and Methods*.

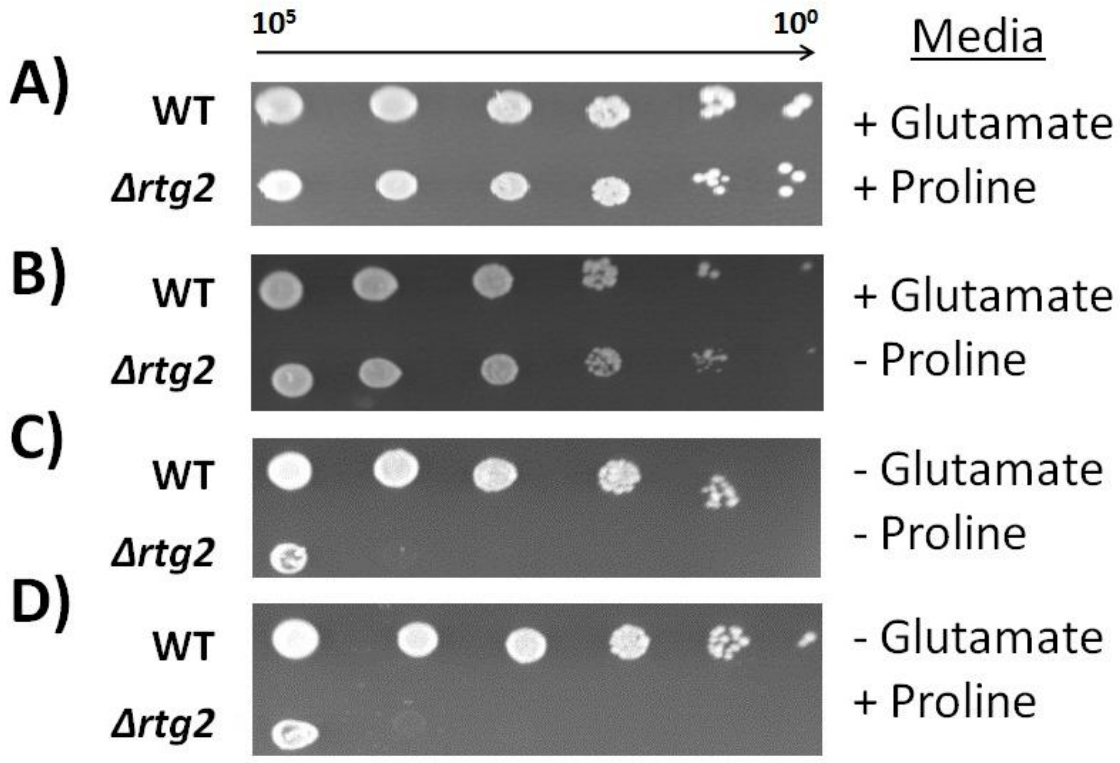


Figure 7. Glutamate auxotrophy of *rtg2*Δ

A 10-fold serial dilution of wild type (WT) and *rtg2*Δ mutant cells were spotted to selection plates and placed at 30°C. Growth was scored after 3 days.

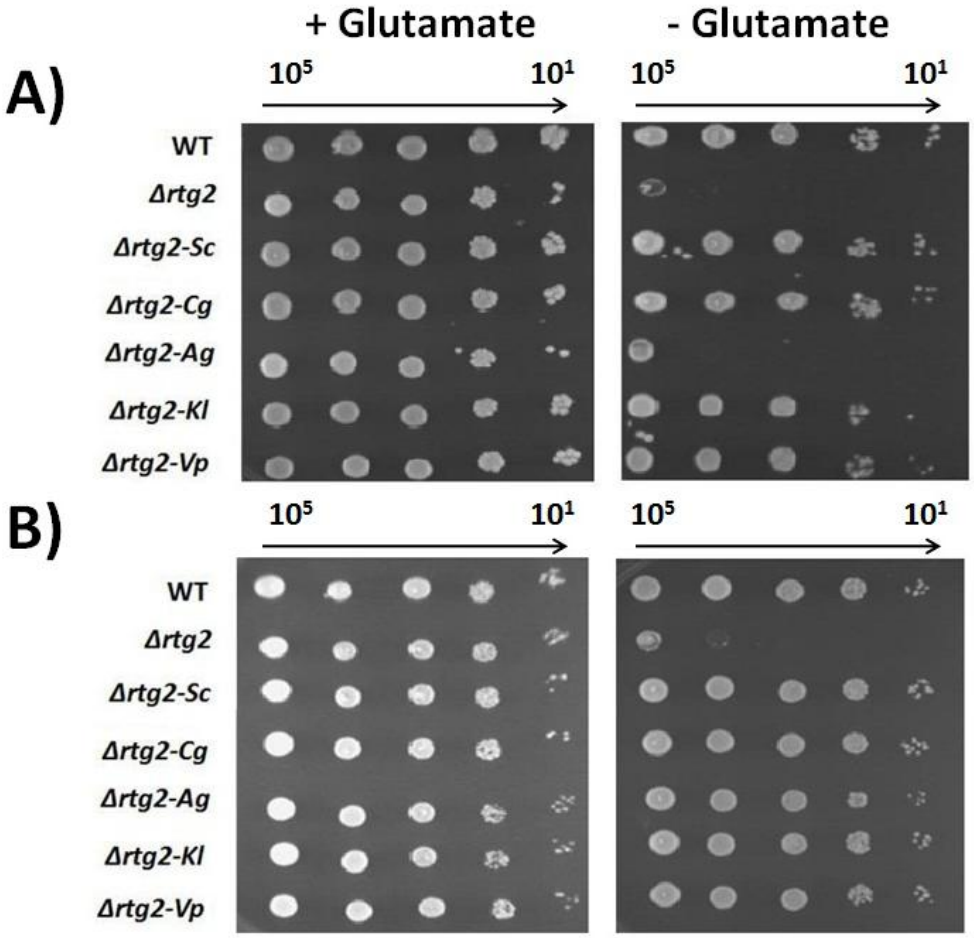


Figure 8. Complementation testing of *rtg2*Δ glutamate auxotrophy by Rtg2p homologs

A) Complementation profile for Rtg2p homologs expressed from the native *RTG2* promoter.

B) Complementation profile for Rtg2p homologs expressed from the constitutive *GPD* promoter.

10-fold serial dilutions of cells expressing the indicated RTG2 genes were spotted to media with and without glutamate. Growth was scored after 3 days of growth at 30^{o}C for 3 days.

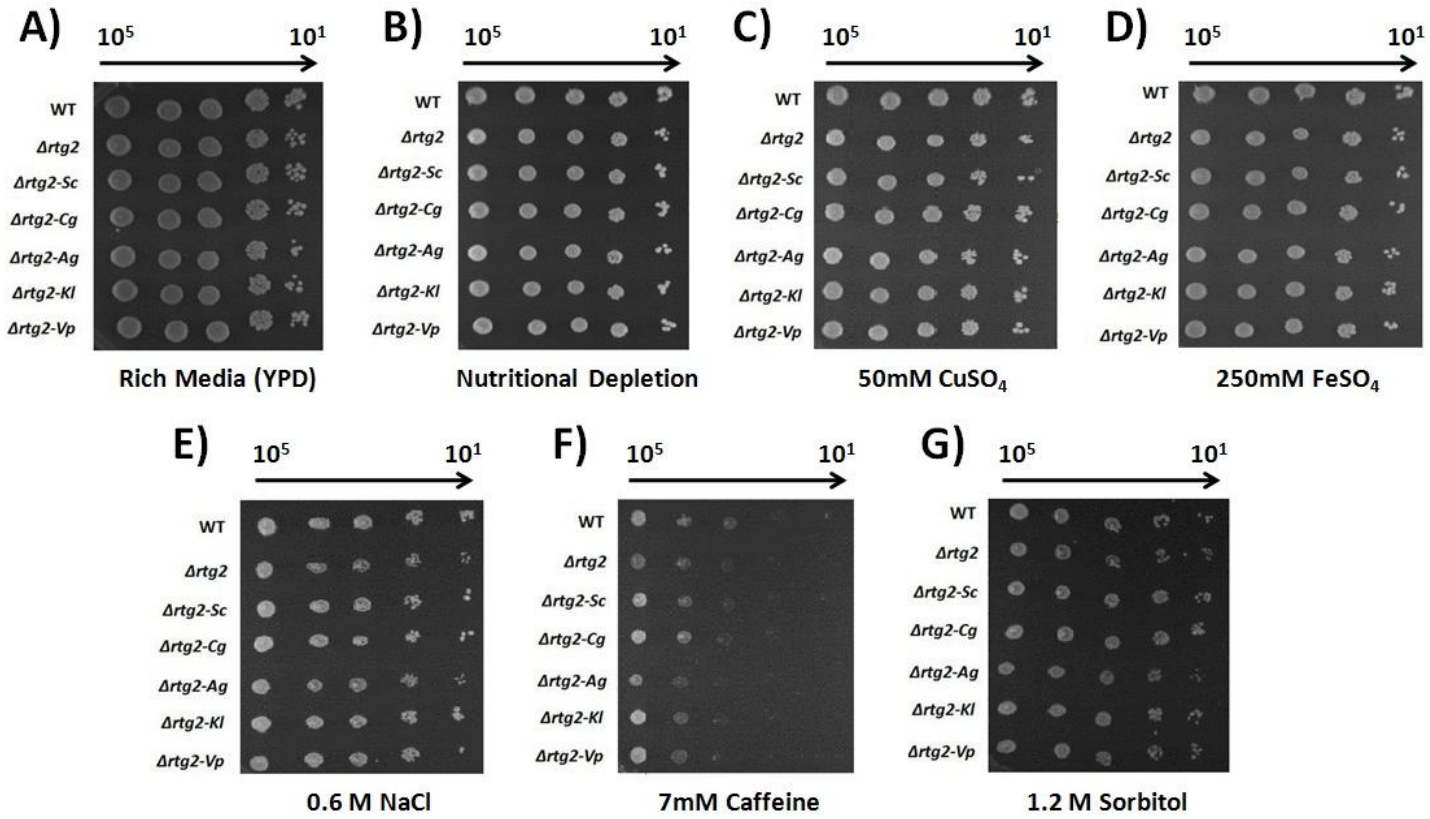
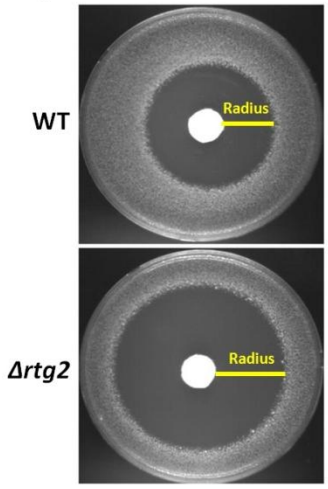


Figure 9. The role of Rtg2p in general stress response

A 10-fold serial dilution of cells expressing the indicated *RTG2* genes were spotted to different plates to test for sensitivity to the following stress conditions: A) Rich media (YPD); B) Nutritional stress (YNB media), C) Heavy metal (50 mM $CuSO_4$) D) Heavy metal (250 mM $FeSO_4$), E) Salt stress (0.6 M NaCl), F) Cytotoxic agent (7 mM caffeine), and G) Osmotic stress (1.2 M sorbitol).

A)



B)

Strain	Radius of Growth Inhibiton Zone (cm), n = 6 (*Under RTG2 promoter*)
WT	**1.36 ± 0.05**
Δrtg2	**1.93 ± 0.08**
Δrtg2-Sc	**1.40 ± 0.03**
Δrtg2-Cg	**1.44 ± 0.05**
Δrtg2-Ag	**1.57 ± 0.11**
Δrtg2-Kl	**1.39 ± 0.06**
Δrtg2-Vp	**1.41 ± 0.06**

Strain	Radius of Growth Inhibiton Zone (cm), n = 6 (*Under GPD promoter*)
WT	**1.33 ± 0.05**
Δrtg2	**1.94 ± 0.18**
Δrtg2-Sc	**1.34 ± 0.06**
Δrtg2-Cg	**1.36 ± 0.09**
Δrtg2-Ag	**1.35 ± 0.09**
Δrtg2-Kl	**1.38 ± 0.09**
Δrtg2-Vp	**1.31 ± 0.07**

C)

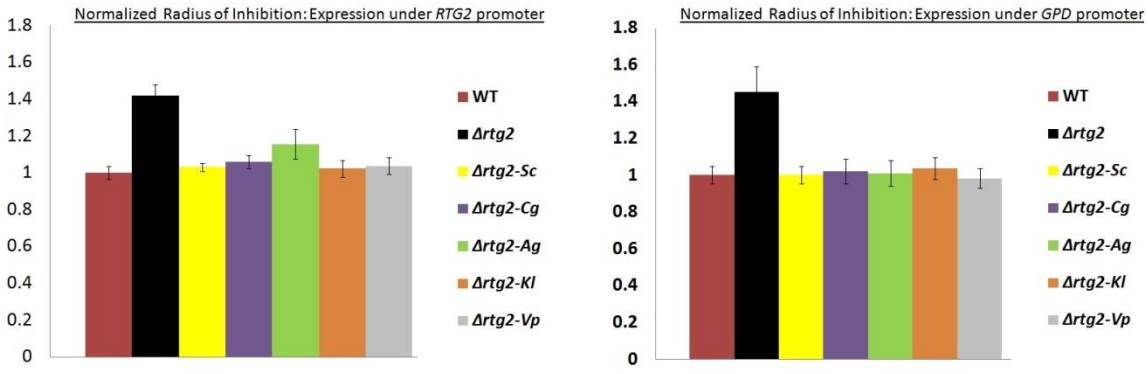


Figure 10. Sensitivity to oxidative stress as measured by plate overlay assay

A) Plate overlay assay for WT and *rtg2*Δ mutant. ~3x10^5 exponentially growing cells were plated to selective plates. 10µl of concentrated H_2O_2 was spotted onto a sterile Whatmann disc at the center of the plate and cells were grown at 30^{o}C for 3 days before scoring growth inhibition zones.

B) Growth inhibition zone radius measured from cells expressing the indicated *RTG2* gene under both the native *RTG2* promoter and constitutive *GPD* promoter

C) Radius of growth inhibition values were normalized against that of WT and plotted.

A)

Strain	Radius of Growth Inhibiton Zone (cm), n = 6
WT (+ glutamate)	1.61 ± 0.05
WT (- glutamate)	2.46 ± 0.08
Δrtg2 (+ glutamate)	2.05 ± 0.05

B)

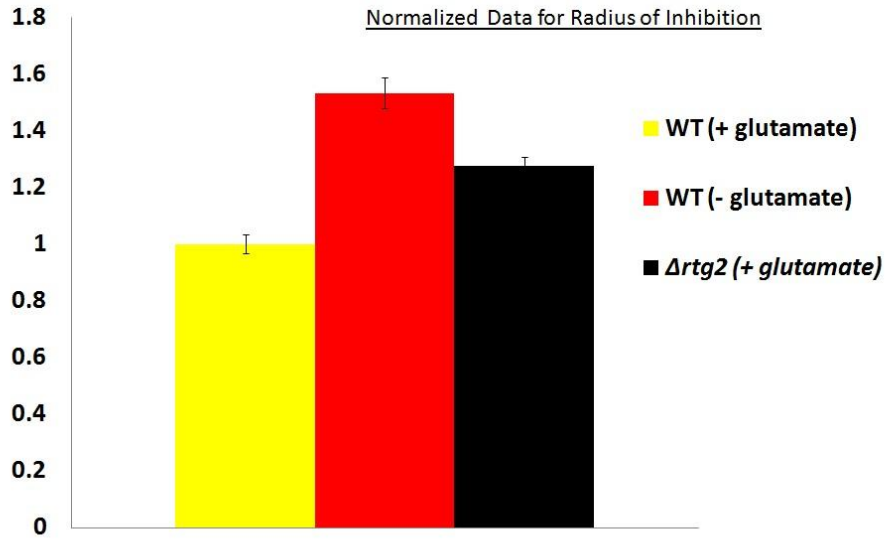


Figure 11. H_2O_2 mediated growth sensitivity with induction of retrograde signaling

Overlay assay for WT and *rtg2*Δ mutant. ~$3x10^5$ exponentially growing cells were plated on selective plates with or without glutamate. 10µl of concentrated H_2O_2 was spotted onto a sterile Whatmann disc and cells grown at 30^oC for 3 days

A) Radius of growth inhibition zones measured from WT and *rtg2*Δ cells grown in the presence and the absence of glutamate.

B) Radius of growth inhibition values were normalized against that of WT (+glutamate) and plotted.

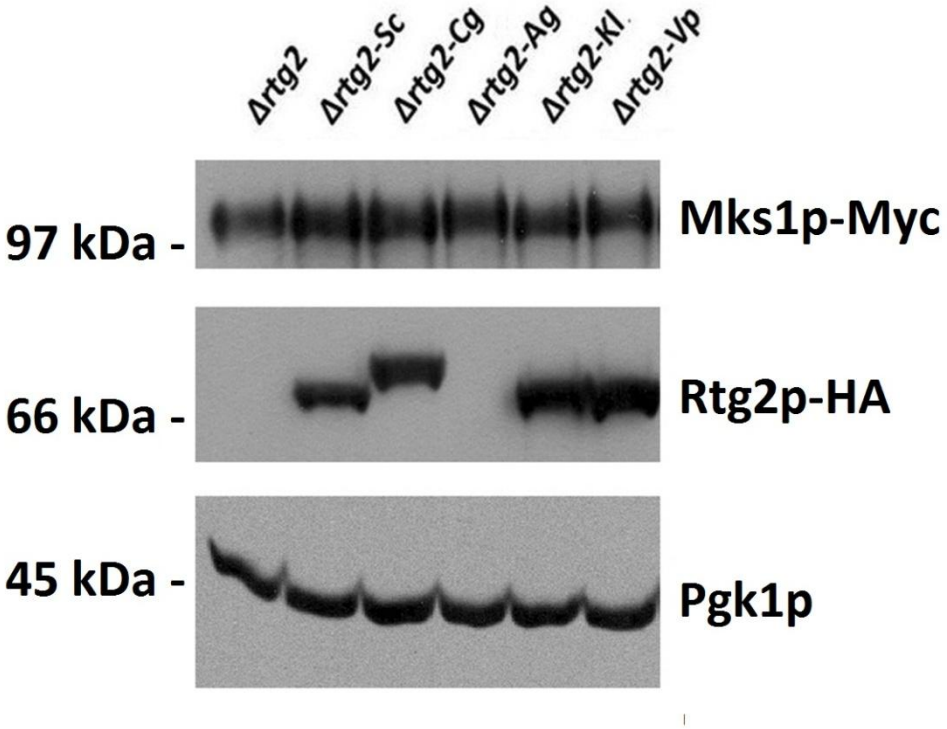


Figure 12. Steady state levels of Mks1p and Rtg2p

Basal steady state protein levels for strains expressing Rtg2p homologs. Whole cell extracts were prepared by alkaline lysis from $5x10^6$ exponentially growing cells. Proteins were separated by SDS-PAGE followed by western blot analysis using antibodies against Myc to detect Mks1p; HA to detect Rtg2p; and Pgk1p. Pgk1p is used as a loading control.

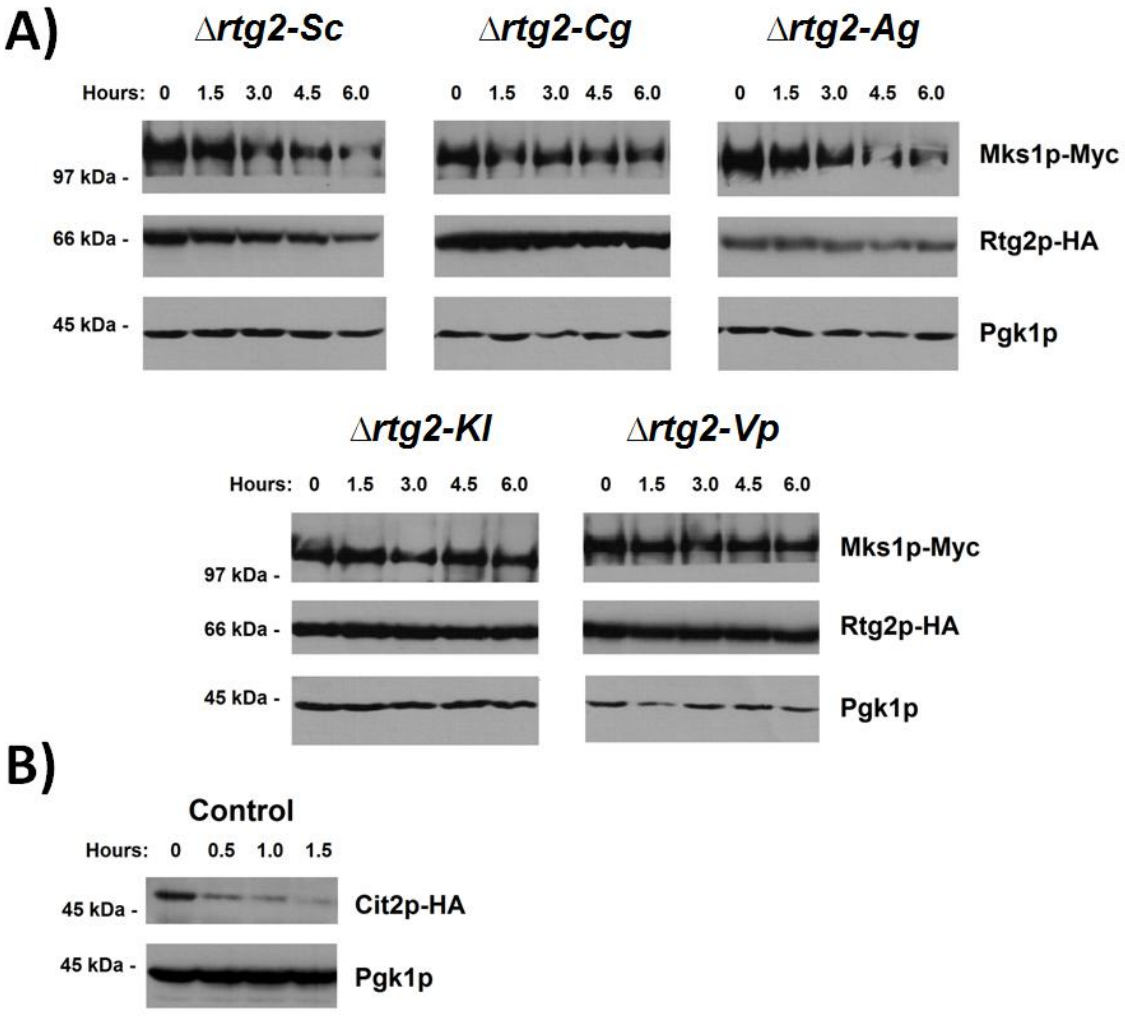


Figure 13. Half-lives of Rtg2p and Mks1p proteins

A) The stability of Mks1p and Rtg2p proteins in *rtg2*Δ cells expressing different Rtg2p homologs from the native Rtg2p promoter was determined using a cycloheximide chase assay system. An equal number of cells were collected at 90 minute intervals and processed for SDS-PAGE followed by Western blot analysis using antibodies against Myc to detect Mks1p; HA to detect Rtg2 and Cit2p, and anti-Pgk1p to detect Pgk1p. Pgk1p levels were used as an internal control to show equal lane loading.
* *A. gossypii* Rtg2p protein was detected by 14 fold higher exposure time.

B) The half-life of Cit2p in WT cells as a control for cyclohexomide treatment.

Table 5. Calculated half-lives of Rtg2p homologs and Mks1p expressed in *rtg2*Δ

Expressed Rtg2p	**Half-lives (hour) , n=3**	
	Rtg2p	**Mks1p**
S. cerevisiae	2.95 ± 0.1	2.895 ± 0.8
C. glabrata	> 6	6
A. gossypii	2.21 ± 0.2	3.065 ± 0.7
K. lactis	>6	> 6
V. polyspora	>6	> 6

NIH Image J software was used to determine the change in Rtg2p and Mks1p protein levels obtained by western blot analysis. Data was normalized to Pgk1p for each time point.

Table 6. Codon usage profiles for different yeast

S. cerevisiae				*C. glabrata*				*A. gossypii*				*K. lactis*				*V. polyspora*			
Codon	Amino Acid	Hit Number	Fraction	Codon	Amino Acid	Hit Number	Fraction	Codon	Amino Acid	Hit Number	Fraction	Codon	Amino Acid	Hit Number	Fraction	Codon	Amino Acid	Hit Number	Fraction
CGA	R	9053	6.997650187	CGA	R	6513	5.5667618	CGA	R	9957	7.1335946	CGA	R	7346	6.85401855	CGA	R	4394	4.16429736
CGC	R	7646	5.910088736	CGC	R	5738	4.9043573	CGC	R	38820	27.812207	CGC	R	4564	4.2583366	CGC	R	1811	1.71632738
CGG	R	5299	4.095940389	CGG	R	6208	5.3060736	CGG	R	31472	22.547804	CGG	R	4918	4.58862826	CGG	R	1325	1.25573373
CGU	R	18271	14.12283956	CGU	R	15095	12.90193	CGU	R	17457	12.506896	CGU	R	16939	15.8045494	CGU	R	16204	15.3569127
AGA	R	61538	47.56670686	AGA	R	60420	51.641908	AGA	R	21951	15.726578	AGA	R	54345	50.7053686	AGA	R	66582	63.1013306
AGG	R	27565	21.30677426	AGG	R	23024	19.678969	AGG	R	19922	14.272921	AGG	R	19066	17.7890985	AGG	R	15200	14.4053982
CUA	L	39442	14.22758656	CUA	L	41066	16.74284	CUA	L	30368	13.069713	CUA	L	34082	14.2754822	CUA	L	30964	12.603643
CUC	L	16088	5.803291225	CUC	L	13994	5.7054327	CUC	L	36843	15.856409	CUC	L	15352	6.43029173	CUC	L	5222	2.1255724
CUG	L	31051	11.2007705	CUG	L	36897	15.043115	CUG	L	71407	30.731987	CUG	L	15829	6.63008649	CUG	L	12691	5.16576778
CUU	L	35756	12.89796625	CUU	L	32349	13.18887	CUU	L	29650	12.760701	CUU	L	35456	14.8509916	CUU	L	22057	8.9781215
UUA	L	77615	27.99741723	UUA	L	54303	22.139639	UUA	L	19492	8.3889238	UUA	L	59198	24.7954931	UUA	L	105005	42.7414267
UUG	L	77270	27.87296823	UUG	L	66666	27.180104	UUG	L	44594	19.192267	UUG	L	78828	33.0176548	UUG	L	69736	28.3854686
UCA	S	55729	21.27231572	UCA	S	49771	21.750778	UCA	S	22451	12.119167	UCA	S	48295	22.2194106	UCA	S	72220	28.2713454
UCC	S	40978	15.64171174	UCC	S	32332	14.129637	UCC	S	33681	18.18118	UCC	S	31491	14.4882795	UCC	S	26735	10.4657217
UCG	S	25390	9.691616504	UCG	S	20273	8.8596476	UCG	S	39845	21.50854	UCG	S	23730	10.9176232	UCG	S	15611	6.11110459
UCU	S	68211	26.03681975	UCU	S	58949	25.761721	UCU	S	31754	17.140976	UCU	S	60368	27.7739182	UCU	S	78158	30.5958435
AGC	S	29011	11.07378836	AGC	S	26841	11.729976	AGC	S	38528	20.797616	AGC	S	19688	9.05799268	AGC	S	16430	6.43171151
AGU	S	42660	16.28374793	AGU	S	40658	17.768241	AGU	S	18993	10.252521	AGU	S	33783	15.5427756	AGU	S	46299	18.1242733
ACA	T	53144	30.82294669	ACA	T	51312	33.25901	ACA	T	28515	22.466908	ACA	T	40890	28.4701721	ACA	T	53384	29.2819922
ACC	T	36398	21.11044735	ACC	T	31424	20.368162	ACC	T	33705	26.556098	ACC	T	30257	21.0668133	ACC	T	38829	21.298338
ACG	T	23770	13.78634357	ACG	T	16699	10.823827	ACG	T	38383	30.241885	ACG	T	18823	13.1057483	ACG	T	9350	5.12862706
ACU	T	59105	34.28026239	ACU	T	54845	35.549002	ACU	T	26317	20.735109	ACU	T	53654	37.3572662	ACU	T	80747	44.2910427
CCA	P	51996	40.74410732	CCA	P	54054	48.255606	CCA	P	29528	26.031455	CCA	P	49059	46.3082877	CCA	P	62088	57.3169381
CCC	P	20144	15.78485456	CCC	P	12354	11.028782	CCC	P	24657	21.737252	CCC	P	11068	10.4474231	CCC	P	5258	4.85395665
CCG	P	15785	12.36913867	CCG	P	11602	10.357449	CCG	P	35719	31.48935	CCG	P	13767	12.9950916	CCG	P	5623	5.19090876
CCU	P	39691	31.10189945	CCU	P	34006	30.358163	CCU	P	23528	20.741942	CCU	P	32046	30.2491977	CCU	P	35355	32.6381965
GCA	A	47549	29.669356	GCA	A	42881	29.381616	GCA	A	40077	21.974449	GCA	A	42457	30.7891454	GCA	A	42964	33.9496808
GCC	A	35411	22.09555543	GCC	A	30705	21.038747	GCC	A	45959	25.199583	GCC	A	25664	18.6111272	GCC	A	18241	14.4138378
GCG	A	17997	11.22966624	GCG	A	15402	10.553291	GCG	A	61560	33.753701	GCG	A	14206	10.3019667	GCG	A	5803	4.58546684
GCU	A	59306	37.00542234	GCU	A	56957	39.026346	GCU	A	34784	19.072267	GCU	A	55569	40.2977606	GCU	A	59544	47.0510146
GGA	G	32725	22.54921551	GGA	G	26323	19.021433	GGA	G	21570	15.512182	GGA	G	31034	24.7158797	GGA	G	24971	18.8652589
GGC	G	28524	19.6545095	GGC	G	24494	17.699767	GGC	G	55068	39.602451	GGC	G	16643	13.2547008	GGC	G	12784	9.65814226
GGG	G	17677	12.18036616	GGG	G	14633	10.574047	GGG	G	32103	23.087047	GGG	G	15469	12.319712	GGG	G	11156	8.42820987
GGU	G	66201	45.61590882	GGU	G	72936	52.704753	GGU	G	30311	21.79832	GGU	G	62417	49.7097075	GGU	G	83454	63.0483889
GUA	V	35404	21.83383492	GUA	V	32199	20.933861	GUA	V	20482	13.652027	GUA	V	29796	20.1622671	GUA	V	37522	24.0065515
GUC	V	32737	20.18908185	GUC	V	30562	19.869582	GUC	V	36486	24.319298	GUC	V	29892	20.2272281	GUC	V	30636	19.6008932
GUG	V	31271	19.28499186	GUG	V	34585	22.485096	GUG	V	60925	40.608816	GUG	V	31725	21.467577	GUG	V	17179	10.9911132
GUU	V	62740	38.69209137	GUU	V	56467	36.711461	GUU	V	32136	21.419859	GUU	V	56368	38.1429277	GUU	V	70962	45.4014421
AAA	K	125283	58.46504921	AAA	K	92837	48.239543	AAA	K	40814	31.497388	AAA	K	93280	53.6708861	AAA	K	126583	64.5762444
AAG	K	89004	41.53495079	AAG	K	99613	51.760457	AAG	K	88765	68.502612	AAG	K	80520	46.3291139	AAG	K	69438	35.4237556
AAC	N	72287	40.22111682	AAC	N	69372	43.697797	AAC	N	57060	60.53148	AAC	N	59966	43.9288828	AAC	N	50981	28.010615
AAU	N	107437	59.77888318	AAU	N	89382	56.302203	AAU	N	37205	39.46852	AAU	N	76541	56.0711172	AAU	N	131025	71.989385
CAA	Q	79138	68.59971221	CAA	Q	66741	64.449187	CAA	Q	29510	29.771994	CAA	Q	69253	66.9544536	CAA	Q	78053	79.5744637
CAG	Q	36224	31.40028779	CAG	Q	36815	35.550813	CAG	Q	69610	70.228006	CAG	Q	34180	33.0455464	CAG	Q	20035	20.4255363
CAC	H	22561	35.73170732	CAC	H	22179	39.901052	CAC	H	32501	57.860818	CAC	H	16963	31.5086559	CAC	H	13244	25.1137743
CAU	H	40579	64.26829268	CAU	H	33406	60.098948	CAU	H	23670	42.139182	CAU	H	36873	68.4913441	CAU	H	39492	74.8862257
GAA	E	133741	70.09743544	GAA	E	108403	60.809689	GAA	E	56128	37.294848	GAA	E	115541	70.1374936	GAA	E	149529	80.4356129
GAG	E	57052	29.90256456	GAG	E	69863	39.190311	GAG	E	94370	62.705152	GAG	E	49194	29.8625064	GAG	E	36370	19.5643871
GAC	D	59395	34.79863137	GAC	D	58218	35.829989	GAC	D	74632	57.621099	GAC	D	45501	30.5394286	GAC	D	35463	21.8247277
GAU	D	111287	65.20136863	GAU	D	104266	64.170011	GAU	D	54890	42.378901	GAU	D	103490	69.4605714	GAU	D	127027	78.1752723
UAC	Y	42553	43.1935605	UAC	Y	43287	47.163356	UAC	Y	48228	63.8883	UAC	Y	38152	46.3037806	UAC	Y	33127	34.0984653
UAU	Y	55964	56.8064395	UAU	Y	48494	52.836644	UAU	Y	27260	36.1117	UAU	Y	44243	53.6962194	UAU	Y	64024	65.9015347
UGC	C	13812	37.64000545	UGC	C	10361	33.816378	UGC	C	21123	63.533552	UGC	C	9888	32.502794	UGC	C	5583	18.753779
UGU	C	22883	62.35999455	UGU	C	20278	66.183622	UGU	C	12124	36.466448	UGU	C	20534	67.497206	UGU	C	24187	81.246221
UUC	F	52209	40.5406035	UUC	F	50857	46.154335	UUC	F	52160	57.527931	UUC	F	59076	54.7948763	UUC	F	45949	40.0252613
UUU	F	76573	59.4593965	UUU	F	59332	53.845665	UUU	F	38509	42.472069	UUU	F	48737	45.2051237	UUU	F	68851	59.9747387
AUA	I	53521	27.95969115	AUA	I	52348	30.484332	AUA	I	27116	23.312757	AUA	I	37289	23.913629	AUA	I	50512	26.6136978
AUC	I	49540	25.8799929	AUC	I	46874	27.296603	AUC	I	49595	42.638891	AUC	I	50248	32.2243029	AUC	I	42233	22.2516689
AUU	I	88361	46.16031595	AUU	I	72499	42.219065	AUU	I	39603	34.048352	AUU	I	68395	43.8620681	AUU	I	97052	51.1346333
AUG	M	60428	100	AUG	M	58162	100	AUG	M	50638	100	AUG	M	51154	100	AUG	M	52976	100
UGG	W	30180	100	UGG	W	26095	100	UGG	W	26687	100	UGG	W	26816	100	UGG	W	25618	100
UAA	STOP	2797	46.53135917	UAA	STOP	2575	49.500192	UAA	STOP	2096	30.076051	UAA	STOP	2271	44.7399527	UAA	STOP	2960	56.6290415
UAG	STOP	1350	22.45882549	UAG	STOP	1215	23.356401	UAG	STOP	2071	29.71732	UAG	STOP	996	19.6217494	UAG	STOP	1024	19.5905873
UGA	STOP	1864	31.00981534	UGA	STOP	1412	27.143406	UGA	STOP	2802	40.206629	UGA	STOP	1809	35.6382979	UGA	STOP	1243	23.7803711

Table 7. Codon usage differences for *RTG2* from *A. gossypii* and *S. cerevisiae*

Codon	Amino Acid	*RTG2* Frequency (%)	Genomic Frequency (%)	
			A. gossypii	***S. cerevisiae***
UCG	S	4	22	10
CUG	L	5	31	11
CCG	P	5	31	12
GCG	A	6	34	11
GGC	G	7	40	20
GUG	V	7	41	19
GAG	E	11	63	30
CAG	Q	12	70	31
	Total	51		

Codons having more than a 4% usage frequency in *RTG2* from *A. gossypii* were analyzed for genomic frequencies both in *A. gossypii* and *S. cerevisiae* genomes. Codons having genome-wide usage frequencies 2 fold higher in *A. gossypii* than in *S. cerevisiae* are presented.

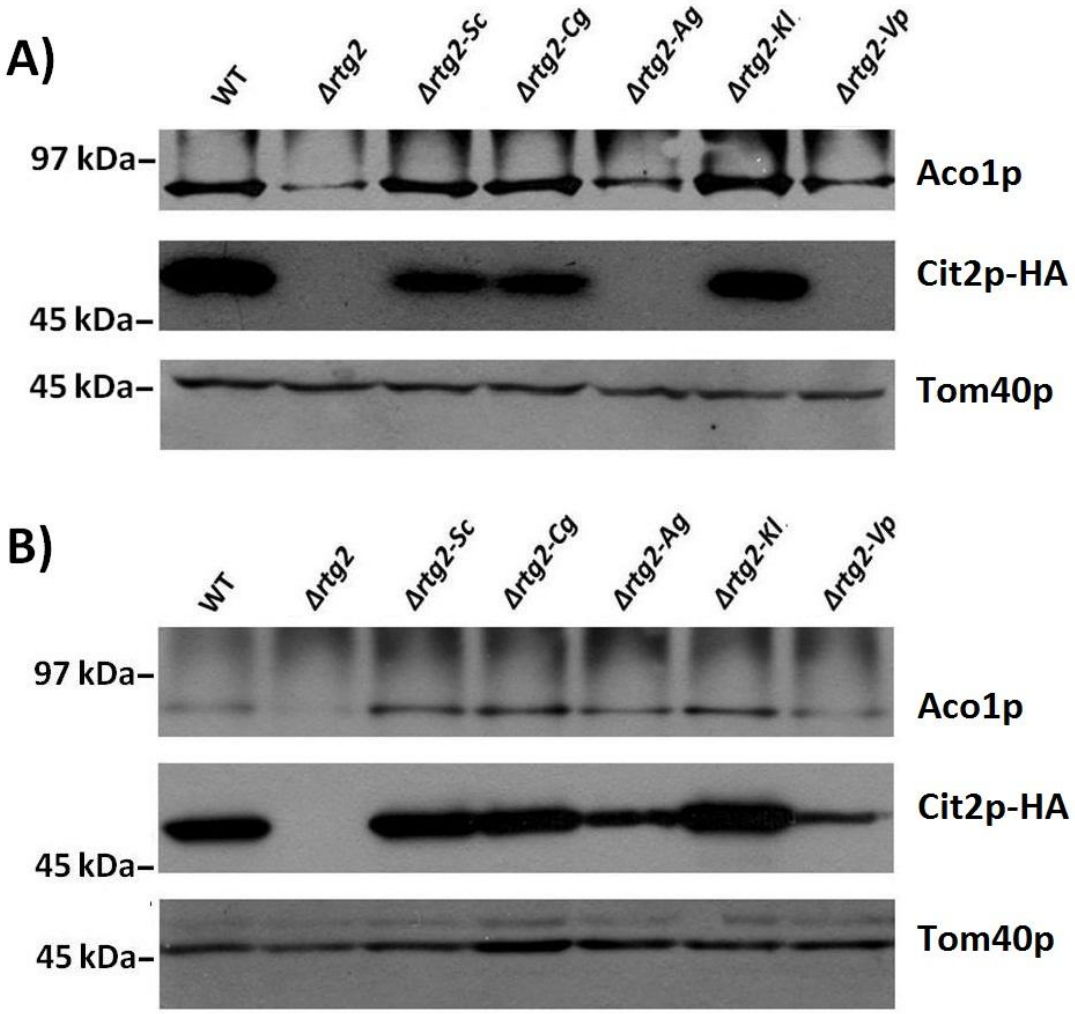


Figure 14. Aco1p and Cit2p protein levels in *rtg2*Δ expressing Rtg2p homologs

A) Aco1p and Cit2p protein levels in *rtg2*Δ cells expressing Rtg2p homologs from the native Rtg2p promoter.

B) Aco1p and Cit2p protein levels in *rtg2*Δ cells expressing Rtg2p homologs from the *GPD* promoter.

$5x10^6$ cells from an exponentially growing culture were processed by alkaline lysis followed by TCA precipitation. The resulting whole cell extracts were separated by SDS-PAGE followed by western blot analysis using antibodies against Aco1p; HA to detect Cit2p; and Tom40p. Tom40p was included to confirm equal lane loading.

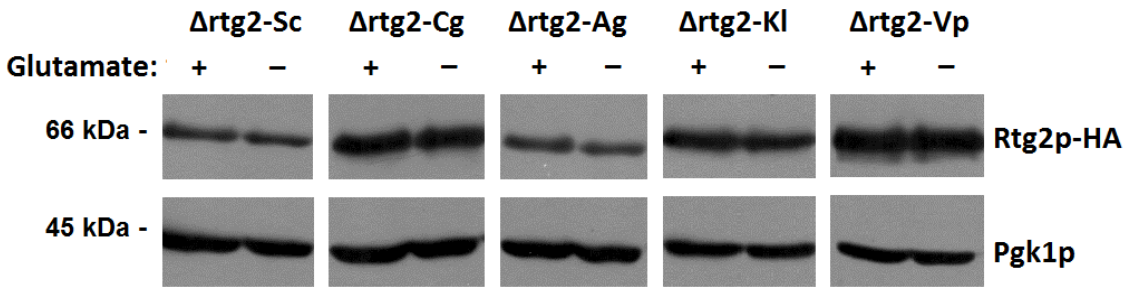
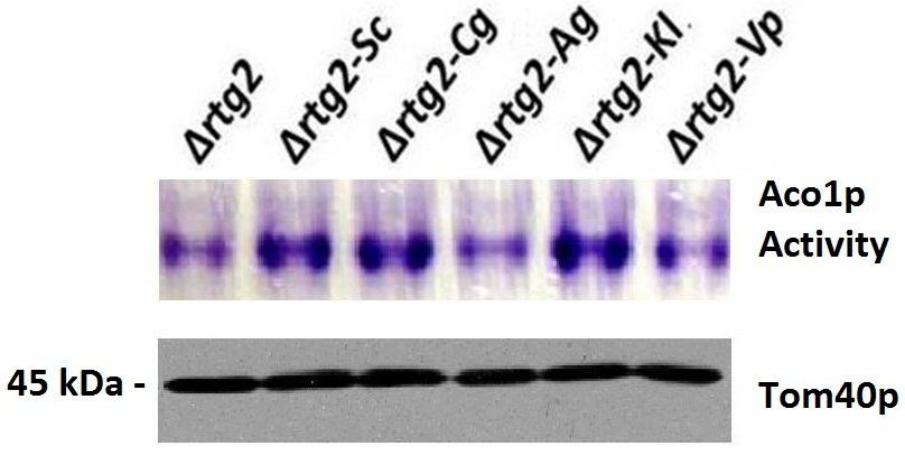


Figure 15. Rtg2p levels with activation of retrograde signaling

$5x10^6$ cells were harvested from an exponentially growing culture grown in selective media with or without glutamat. Protein extracts were prepared by alkaline lysis followed by TCA precipitation and separated by SDS-PAGE. Western blot analysis was carried out using anti-HA to detect Rtg2p-HA and anti-Pgk1p to detect Pgk1p. Pgk1p levels were used to confirm equal loading.



Strain	Aconitase Activity Ratio, n = 3 (Normalized)
Δrtg2-Sc	1.00 ± 0.00
Δrtg2	0.60 ± 0.02
Δrtg2-Cg	1.03 ± 0.04
Δrtg2-Ag	0.56 ± 0.01
Δrtg2-Kl	1.17 ± 0.01
Δrtg2-Vp	0.61 ± 0.02

Figure 16. In-gel aconitase activity in strains expressing Rtg2p homologs

Aconitase activity was detected from crude mitochondrial extracts using an in-gel assay. 200 ng mitochondrial extracts were separated on a 6% Native PAGE at 4^{o}C. In-gel aconitase activities were detected by incubating the gel with activation buffer in the dark. Mitochondrial samples were run on an SDS-PAGE in parallel for detecting Tom40p levels by western blot Tom40p as a loading control.

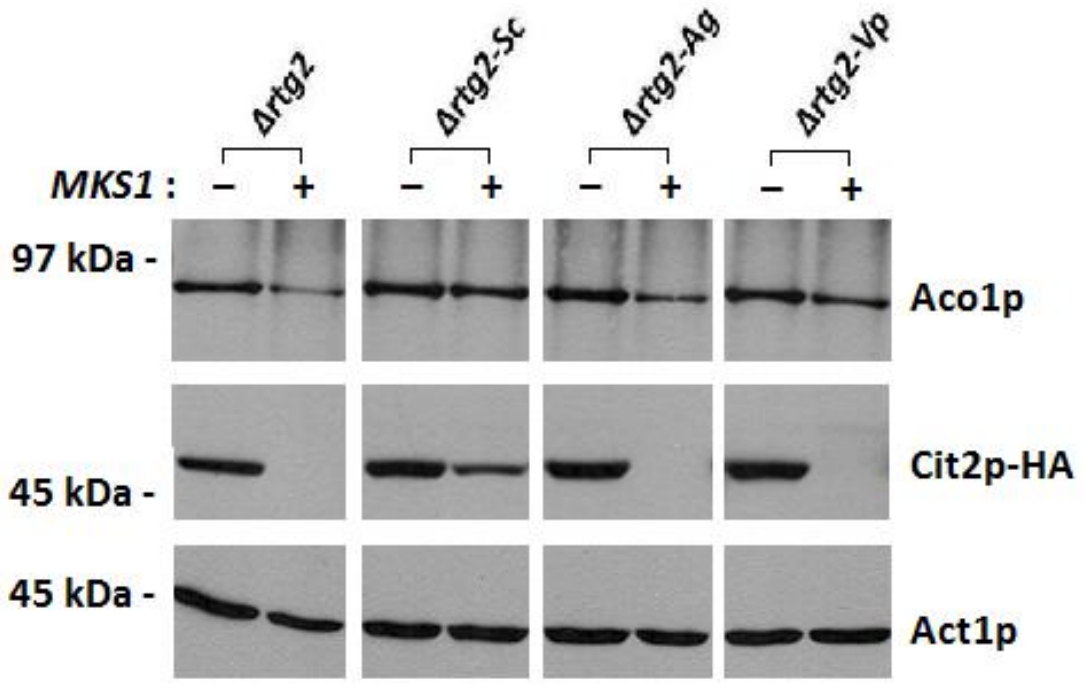


Figure 17. Aco1p and Cit2p expression levels in *rtg2*Δ*mks1*Δ double mutants

Steady state levels of Aco1p and Cit2p in *rtg2*Δ cells expressing Rtg2p homologs from the native *RTG2* promoter. $5x10^6$ cells were harvested from an exponentially growing culture. Cells were processed by alkaline lysis and total cellular protein was separated by SDS-PAGE, transferred to nitrocellulose membrane and analyzed by western blot using antibodies against Aco1p, actin, and HA to detect Cit2p.

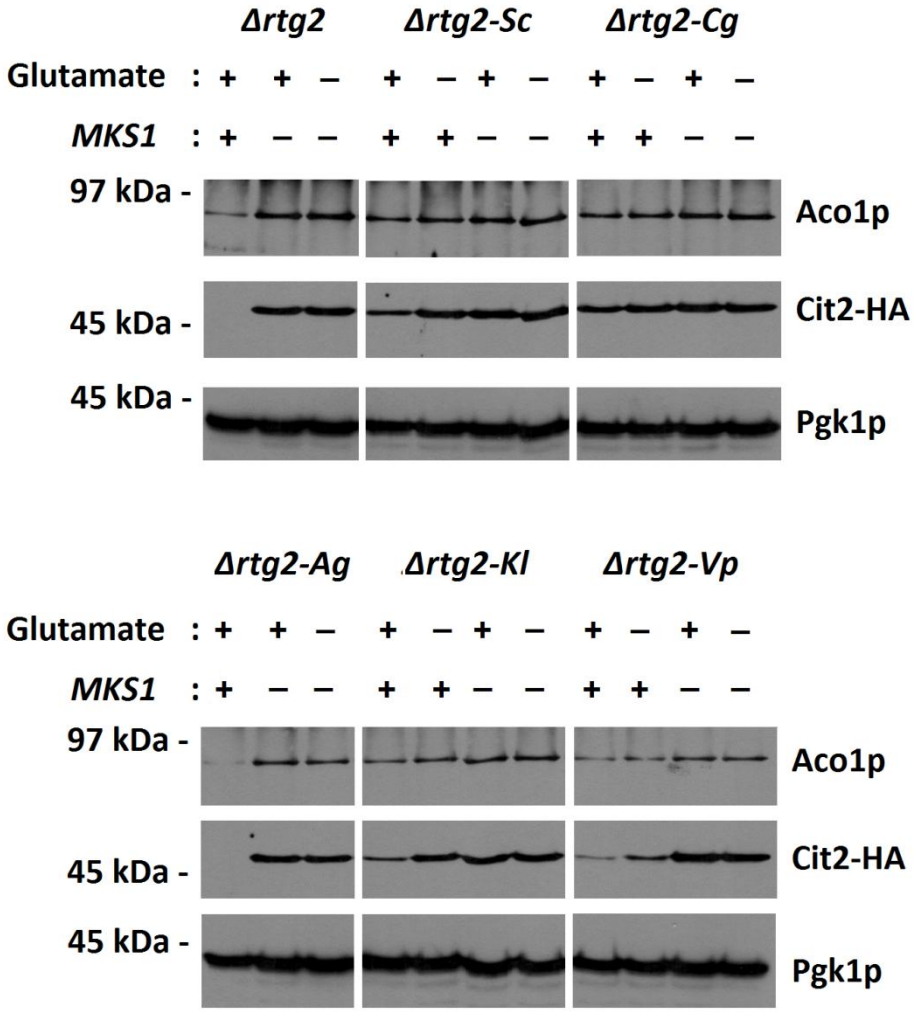


Figure 18. Cit2p and Aco1p protein levels in *mks1*Δ in the presence and absence of glutamate

$5x10^6$ cells grown in the presence or absence of glutamate were harvested by centrifugation. Whole cell extracts were prepared by alkaline lysis and total protein extracts were separated by SDS-PAGE followed by western blot analysis using antibodies against Aco1p, Pgk1p and HA to detect Cit2p. Aco1p, Cit2p and Pgk1p levels were detected in *rtg2*Δ single and *rtg2*Δ*mks1*Δ double mutant strains expressing Rtg2p homologs from the native *RTG2* promoter. Detection of Pgk1p was used as a loading control.

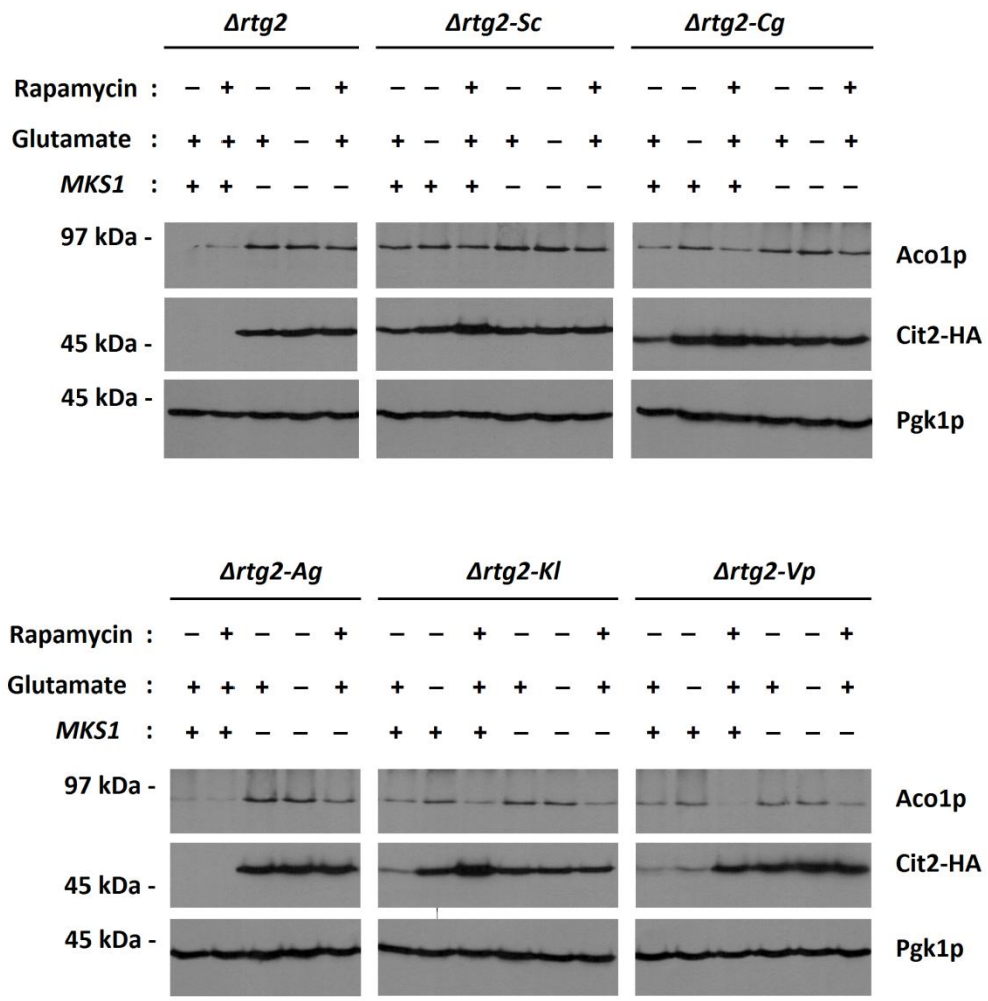


Figure 19. Expression profiles of Cit2p and Aco1p in the presence and absence of glutamate and rapamycin

Whole cell extracts were prepared by alkaline lysis from $5x10^6$ cells grown in the presence and absence of glutamate. For rapamycin treatment, exponentially growing cells were resuspended in selective media ($5x10^6$ cells/ml) containing 100 µg/ml rapamycin for 45 min at 30°C. Total protein extracts were separated by SDS-PAGE followed by western blot analysis using antibodies against Aco1p, Pgk1p and HA to detect Cit2p. Aco1p, Cit2p and Pgk1p levels were detected in *rtg2*Δ single and *rtg2*Δ*mks1*Δ double mutant strain backgrounds expressing Rtg2p homologs from the native *RTG2* promoter. Detection of Pgk1p was used to confirm equal loading.

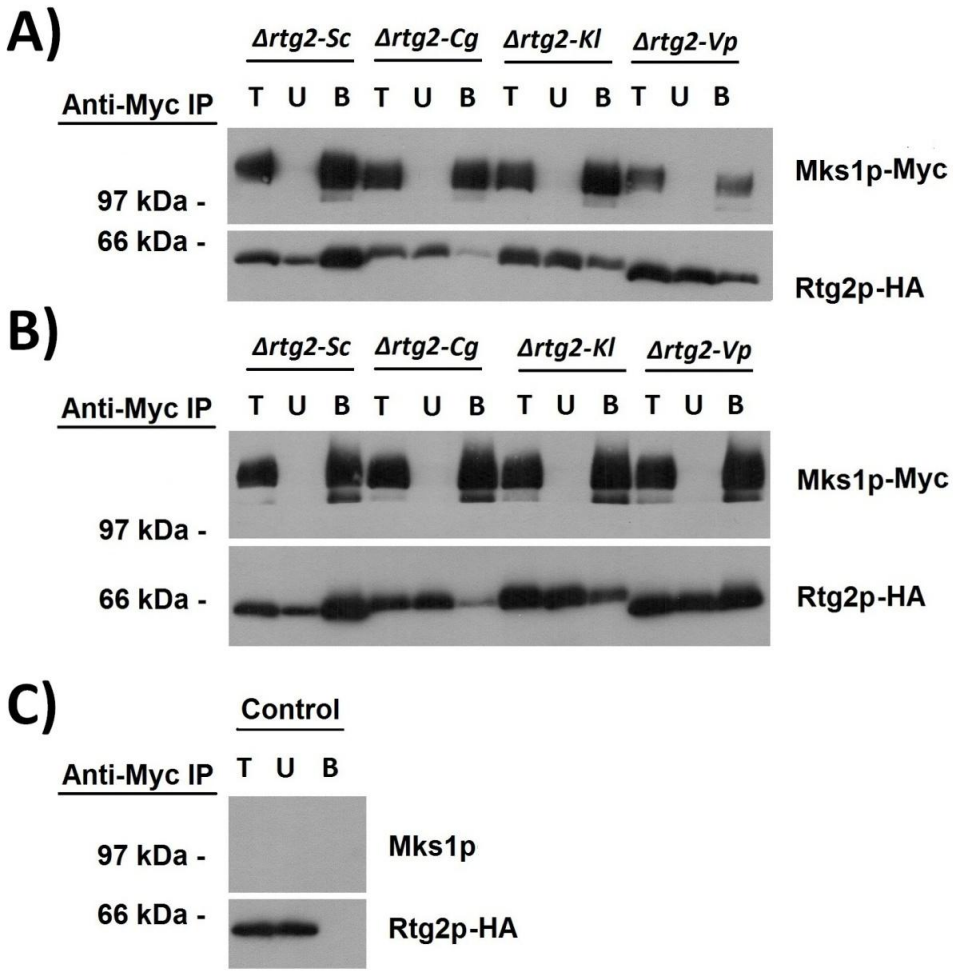


Figure 20. Mks1p and Rtg2p homolog interaction as determined by immunoprecipitation

Co-immunoprecipitation of Rtg2p by Mks1p-Myc pull down was carried out using total protein lysates isolated from cells grown in the presence (A) or absence (B) of glutamate. The specificity of Rtg2p-Mks1p interaction was confirmed using cells expressing an untagged version of Mks1p (C).

Harvested cells were processed by glass bead lysis followed by immunoprecipitation of Mks1p using α-Myc affinity beads. Total (T), unbound (U), and immunoprecipitated (B) Mks1p and Rtg2p levels were detected by western blot using antibodies to Myc and HA to recognize Mks1p and Rtg2p, respectively. Total and unbound samples represent 10% of the total lysates while bound is 87% of total lysates.

Table 8. Relative affinity of Rtg2p homologs for Mks1p

Ratio	Glu	Strains			
		Δrtg2-Sc	*Δrtg2-Cg*	*Δrtg2-Kl*	*Δrtg2-Vp*
Bound Rtg2p / Total Rtg2p	+	0.19± 0.01	0.024 ± 0.04	0.033 ± 0.01	0.051 ± 0.01
	-	0.18 ± 0.01	0.032 ± 0.05	0.068 ± 0.04	0.085 ± 0.01
Bound Rtg2p / Bound Mks1	+	0.70 ± 0.01	0.11 ± 0.03	0.33 ± 0.01	0.26 ± 0.01
	-	1.32 ± 0.03	0.28 ± 0.04	0.68 ± 0.04	0.57 ± 0.04
Bound Rtg2p / Bound Mks1 (Normalized)	+	1.00	0.16	0.47	0.37
	-	1.00	0.21	0.52	0.43
Fold Change		1.89	2.55	2.06	2.19
Fold Change (Normalized)		1.00	1.35	1.09	1.16

Autoradiographs were scanned and images were analyzed using NIH Image J software. Ratios were calculated using the intensity of each protein band from the same autoradiograph. Fold changes were calculated by dividing the ratio of Rtg2p/Mks1p interaction under inducing condition (- Glutamate) by the ratio of Rtg2p/Mks1p interaction under basal (+ Glutamate) condition. Fold changes were normalized against calculated fold change values for *rtg2*Δ expressing Rtg2p from *S. cerevisiae*. Data was collected from 3 independent experiments.

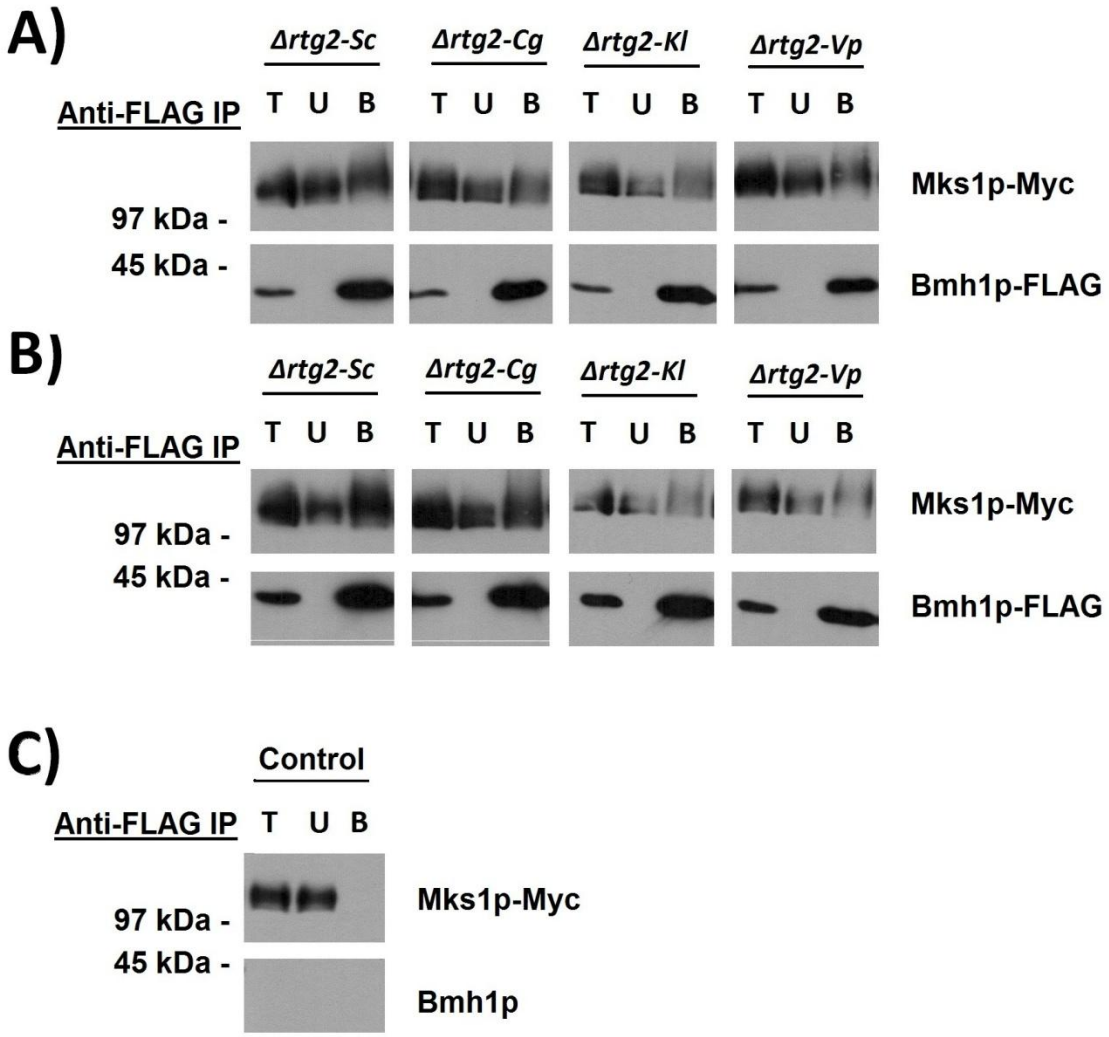


Figure 21. Interaction between Mks1p and Bmh1p as determined by immunoprecipitation

Co-immunoprecipitation of Mks1p by Bmh1p-FLAG pull down was carried out using cell extracts isolated from cells grown in the presence (A) or absence (B) of glutamate. The specificity of Bmh1p-Mks1p interaction was confirmed using cells expressing an untagged version of Bmh1p (C).

Harvested cells were processed by glass bead lysis followed by immunoprecipitation of Bmh1p using α-FLAG affinity beads. Total (T), unbound (U), and immunoprecipitated (B) Bmh1p and Mks1p levels were detected by western blot using antibodies to FLAG and Myc to recognize Bmh1p and Mks1p, respectively. Total and unbound samples represent 10% of the total lysates while bound is 87% of the total lysates.

Table 9. Relative affinity of Bmh1p-Mks1p interaction in cells expressing Rtg2p homologs

Ratio	Glu	Strains			
		Δrtg2-Sc	*Δrtg2-Cg*	*Δrtg2-Kl*	*Δrtg2-Vp*
Bound Mks1p / Total Mks1p	+	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
	-	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
Bound Mks1p / Bound Bmh1	+	0.59 ± 0.02	0.59 ± 0.08	0.62 ± 0.01	0.57 ± 0.01
	-	0.15 ± 0.01	0.25 ± 0.02	0.25 ± 0.03	0.25 ± 0.02
Bound Mks1p / Bound Bmh1 (Normalized)	+	1.00	1.00	1.05	0.97
	-	1.00	1.67	1.67	1.67
Fold Change		3.933333	2.36	2.48	2.28
Fold Change (Normalized)		1.00	0.60	0.63	0.58

Autoradiographs were scanned and images analyzed using NIH Image J software. Ratios were calculated using the intensity of each protein band from the same autoradiograph. Fold changes were calculated by dividing the ratio of Mks1p/Bmh1p interaction under basal condition (+ Glutamate) by the ratio of Mks1p/Bmh1p interaction under inducing (- Glutamate) condition. Fold changes were normalized against calculated fold change values for *rtg2*Δ expressing Rtg2p from *S. cerevisiae*. Data was collected from 3 independent experiments.

CHAPTER IV

DISCUSSION

Fungal Rtg2p homologs can rescue *rtg2*Δ phenotypes

Rtg2p, a central component of the retrograde signaling pathway, is responsible for activation of this pathway in *Saccharomyces cerevisiae*. In many studies, glutamate auxotrophy has been linked to the absence of Rtg2p as this pathway is responsible for the transcriptional expression of genes coding for proteins responsible for glutamate biosynthesis. In our studies, we used the W303 laboratory stain of *Saccharomyces cerevisiae*. Although the S288C (BY4743) strain background is also commonly used in yeast research, this strain background has a mutation in the *HAP1* gene which affects mitochondrial function [85]. Since our study is dependent on intact mitochondrial function, we carried out our work using strains from the W303 background. In this strain background, our *rtg2*Δ mutant phenotypes were comparable to that described in the BY4743 background. In our hands, *rtg2*Δ mutants were glutamate auxotrophs, lacked Cit2p, and had reduced Aco1p protein which in turn results in incompetency for glutamate biosynthesis as reported in previous studies [15, 49]. Work by Velot et al found the glutamate auxotrophy of *rtg2*Δ mutants could be bypassed by supplementing the media with proline, suggesting that proline could be used for the *de-novo* biosynthesis of glutamate [15]. However, in our studies we found that the addition of proline had no effect on the glutamate auxotrophy of *rtg2*Δ cells. The use of different strain backgrounds

to test the role of proline in glutamate auxotrophy is the most likely explanation for these differences.

Our major goal was to analyze various fungal Rtg2p homologs in *rtg2*Δ mutant background of *S. cerevisiae* to evaluate the functional role of Rtg2p in retrograde signaling. We expressed Rtg2p homologs from *C. glabrata, A. gossypii, K. lactis* and *V. polyspora* in our *S. cerevisiae rtg2*Δ shuffle strain. Our initial results showed that *rtg2*Δ dependent glutamate auxotrophy can be bypassed by expressing *C. glabrata, K. lactis* and *V. polyspora* Rtg2p homologs. These data suggest that Rtg2p homologs belonging to *C. glabrata, K. lactis* and *V. polyspora* are capable of receiving and delivering the mitochondrial signal to the nucleus. However, the *A. gossypii* Rtg2p homolog was not able to rescue the glutamate auxotrophy of *rtg2*Δ. The lack of complementation was most likely due to reduced *A. gossypii* protein levels. The low levels of Rtg2p can be explained by altered post-translational processing or mRNA instability.

Given that the calculated protein half-life for the *A. gossypii* Rtg2p homolog was similar to that of *S. cerevisiae* Rtg2p, the low levels of *A. gossypii* Rtg2p is not likely due to proteolysis. Interestingly, Rtg2p homologs showing longer half-lives also showed longer half-lives for Mks1p. It is tempting to speculate that Rtg2p plays a role in increasing the stability of Mks1p, although it is unclear whether this increase in stability can be attributed to the functional state of the retrograde signaling pathway.

In complementation based studies, evolutionary differences can be an important factor especially for the expression of recombinant protein. Although a universal codon system is often utilized for a large group of organism, the codon preferences for the same

amino acid can significantly differ from organism to organism. Given that the reduction in *A. gossypii* Rtg2p level was not due to increased degradation, codon usage profiles for these fungi were determined using a computational approach. We found that *A. gossypii* had a significantly different codon preference compared to those of other fungi suggesting that the low levels of *A. gossypii* Rtg2p protein could be due to reduced translation rate. However, since expression of *A. gossypii* Rtg2p from the stronger *GPD* promoter was sufficient to complement the glutamate auxotrophy of *rtg2*Δ, the reduced protein level is likely due to altered mRNA stability, mRNA processing, mRNA 2^{o} structure changes or ribosome loading capabilities.

It has been shown that the glutamate auxotrophy phenotype of *rtg2*Δ cells is due to lack of expression of *CIT2* [15]. In the absence of glutamate, or any good nitrogen source, cells activate the GATA factor dependent pathway to prevent nitrogen starvation [34]. The inability of *rtg2*Δ mutants to grow in the absence of glutamate suggests that expression of genes by the retrograde signaling pathway and the GATA dependent pathway are required. Nitrogen catabolite repression is under the control of the TOR pathway which also regulates other stress responses by affecting the general stress response transcription factors Msn2p and Msn4p. Under normal cellular conditions, TORC1, the rapamycin sensitive complex in the TOR pathway, functions as an inhibitor of several factors related to stress response. To date, studies have suggested that the TOR pathway also has an inhibitory effect on steps of the retrograde signaling pathway [29]. In our study *rtg2*Δ mutants showed no significant difference in terms of growth characteristics when tested under several stress conditions known to involve the TOR

pathway. This data suggests that retrograde signaling has no direct overlap with the TOR pathway for stress conditions other than nitrogen starvation.

Interestingly, sensitivity to exogenous H_2O_2 is significantly increased in our *rtg2*Δ mutant compared to WT cells. Expressing Rtg2p homologs in *rtg2*Δ rescued this H_2O_2 sensitive phenotype suggesting that Rtg2p has a functional role in the cellular defense mechanism against oxidative stress. However, cells constitutively expressing Rtg2p did not increase their resistance to oxidative stress suggesting that Rtg2p activity is not limiting under these conditions. Moreover, under conditions that activate retrograde signaling (i.e. -glutamate), WT cells showed higher sensitivity to H_2O_2 treatment when compared to WT and *rtg2*Δ mutant cells grown under non-inducing conditions. Together these data suggest that Rtg2p has a role in controlling oxidative stress under basal metabolic conditions. The increased sensitivity of WT cells to H_2O_2 in the absence of glutamate is likely due to the combined effect of increased peroxisomal activities (e.g. β-oxidation of fatty acids) which produces H_2O_2 as a byproduct, and the presence of exogenous H_2O_2. It is likely that the peroxisomal H_2O_2 produced as a result of induction of retrograde signaling and the added H_2O_2 overwhelms the oxidative stress response of the cell.

Studies have shown that mitochondrial retrograde signaling is key for the biosynthesis of α-ketoglutarate in cells with mitochondrial incompetency [8, 15]. In addition to other early TCA cycle genes and peroxisomal glyoxylate genes, expression of *CIT2* and *ACO1* are regulated by retrograde signaling pathway. In our study, we analyzed expression of Cit2p and Aco1p in the *rtg2*Δ mutants expressing different Rtg2p

homologs. As shown in previous studies [8, 29], *rtg2*Δ mutants lose expression of Cit2p. *rtg2*Δ cells expressing either *A. gossypii* or *V. polyspora* Rtg2p homologs were unable to rescue loss of Cit2p expression. However, expression of these Rtg2p homologs from the strong *GPD* promoter resulted in Cit2p protein levels at ~50% of WT. Interestingly, elevated expression of complementing Rtg2p homologs from the *GPD* promoter, including *S. cerevisiae* Rtg2p, did not alter the expression level of Cit2p suggesting that the expression level of Rtg2p is not a limiting regulatory factor in the transmission of retrograde signaling. Therefore, if Rtg2p activity is regulated it is most likely post-translationally.

Fungal Rtg2p homologs are functionally active in retrograde signaling

In this study we also analyzed the changes in Cit2p and Aco1p expression levels in the presence and absence of glutamate to determine whether expression of each Rtg2p homolog is capable of sensing and transmitting the retrograde activation signal. We detected a major increase in Cit2p levels upon removal of glutamate from the media for WT cells as shown previously [12], as well as *C. glabrata* and *K. lactis* Rtg2p homologs. The results for Cit2p expression in cells containing *V. polyspora* were harder to interpret. In general, we detected no expression of Cit2p for cells grown in the presence of glutamate; however for some repeats we detected a very low level of Cit2p suggesting that the *V. polyspora* Rtg2p homolog might show residual function under non-induced conditions.

To confirm that the changes in Cit2p and Aco1p expression were related to glutamate levels and were regulated through the retrograde signaling pathway, we deleted

MKS1. It is known that deletion of *MKS1* results in the constitutive activation of retrograde signaling [80]. Therefore in *rtg2*Δ*mks1*Δ cells the level of Cit2p and Aco1p should parallel that detected in the absence of glutamate if expression is controlled by this pathway. Our results confirmed that the increase in Cit2p and Aco1p levels were linked to the absence of glutamate as the deletion of *MKS1* in *rtg2*Δ cells expressing Rtg2p homologs resulted in glutamate independent expression of Cit2p and Aco1p. As expected, Cit2p protein levels in the *rtg2*Δ *mks1*Δ mutant expressing the *V. polyspora* Rtg2p homolog returned to WT levels suggesting that in the presence of Mks1p, the low induction of Cit2p was a result of the limited functionality of *V. polyspora* Rtg2p in transmitting the retrograde signal to downstream components of the pathway.

Overall, these results show that Rtg2p from *C. glabrata* and *K. lactis* are fully functional in mediating retrograde signaling in *S. cerevisiae*.

Both Mks1p and Rtg2p are required for TOR dependent expression of Cit2p

Glutamate is not the only activator of retrograde signaling. We also tested expression profiles of Cit2p and Aco1p under rapamycin treatment. With rapamycin treatment, Cit2p levels are even higher than that measured in the absence of glutamate for our WT strain. Interestingly, in the absence of *MKS1*, the rapamycin mediated induction of Cit2p expression was reduced to a level seen for cells grown in the absence of glutamate. Note that expression of Cit2p still required Rtg2p as rapamycin failed to restore Cit2p expression in the *rtg2*Δ mutant. Data suggests that TOR dependent GATA factors are involved in the expression of Cit2p. Mks1p is a positive regulator of TORC1 (rapamycin sensitive) dependent GATA factors [86], and it is likely that rapamycin

dependent induction of Cit2p expression is regulated by gene product(s) whose expression is under the control of GATA factors. Deleting *MKS1* likely abolished the contribution of GATA factors on Cit2p expression with rapamycin treatment. Nevertheless, the absence of *MKS1* results in the constitutive activation of Rtg1p/Rtg3p which in turn results in the induction of Cit2p expression [87]. This data suggest that cross talk between the TOR and retrograde signaling pathways are likely to exist however molecular level interaction between these two pathways are unclear.

Aco1p is essential for mitochondrial activities as it is involved in the TCA cycle and mtDNA maintenance [88]. Unlike Cit2p, under basal conditions Aco1p expression is regulated by the Hap complex, and expression of Aco1p by retrograde signaling is dependent on mitochondrial competency [12]. In our studies, Aco1p expression paralleled that seen for Cit2p expression however the magnitude of the changes were minor compared to Cit2p. One difference between Cit2p and Aco1p expression was their dependency on Rtg2p and rapamycin. In the absence of Rtg2p, Aco1p was still expressed to 40-50% of WT levels while Cit2p expression was totally abolished. In the presence of rapamycin, Aco1p levels decrease to basal state levels while Cit2p expression was even higher than that seen in the absence of glutamate. The involvement of Hap in Aco1p expression may explain these differences. The expression level of Hap subunits is regulated through multiple reactions that involve the activation of Snf1p, a serine-threonine protein kinase. Activation of Snf1p is required under glucose repressing conditions for the expression of Aco1p via Hap complex. Snf1p is also involved in the activation of general stress response transcription factors Msn2p/Msn4p [89]. A study by

Mayordomo and colleagues showed that activation of Msn2p/Msn4, along with the TOR complex, results in insensitivity to glucose repression [90]. Note that, the absence of glutamate, or the presence of rapamycin, function as activators for TOR dependent GATA factors. Given that rapamycin affects TORC1, an early component of the pathway, rapamycin treatment would lead to activation of all TOR dependent pathways including the activation of the Msn2p/Msn4p complex. Therefore, a basal level of Aco1p with rapamycin treatment is likely correlated with activation of Msn2p/Msn4p and loss of Hap complex rather than retrograde signaling activation.

Rtg2p and Mks1p-Bmh1p interaction

The exact function of Rtg2p in retrograde signaling is unclear. The dynamic interaction between Rtg2p and Mks1p has been shown to control retrograde signaling [46]. Mks1p is a negative regulator of the pathway when bound to Bmh1p and, under active conditions, more Mks1p is bound to Rtg2p than Bmh1p. To date, no study has determined whether Rtg2p plays a role in influencing the dynamic interaction between Mks1p and Bmh1p. In this study, we measured interaction levels between Rtg2p homologs and Mks1p in the presence and absence of glutamate.

We detected significantly lower binding between Mks1p and fungal Rtg2p homologs when compared to *S. cerevisiae* Rtg2p regardless of glutamate conditions. Despite this, we did observed an increase in interaction between Mks1p and Rtg2p homologs when retrograde signaling was induced. Interestingly, all Rtg2p homologs were able to complement most of the *rtg2*Δ mutant phenotypes despite their apparent lower affinity for Mks1p. Therefore, the function of Rtg2p in the activation of retrograde

signaling is not only dependent on the sequestration of Mks1p but Rtg2p may also have a role in downstream events possible in influencing the Mks1p/Bmh1p complex. In support of this idea, we found that the association between Mks1p and Bmh1p was not altered when Rtg2p homologs were expressed under normal conditions. As previously reported [46], removing glutamate from the media resulted in reduced association between Mks1p and Bmh1p in WT cells. Interestingly, in the absence of glutamate the interaction between Mks1p and Bmh1p for all of our Rtg2p homologs were significantly higher compared to WT.

Taken together, the Mks1p-Rtg2p interaction profiles suggest that Rtg2p may have a role in regulating Mks1p-Bmh1p interaction during activation of retrograde signaling. It is possible that during the interaction between Rtg2p and Mks1p, Mks1p is modified such that it is altered in its ability to interact with Bmh1p.

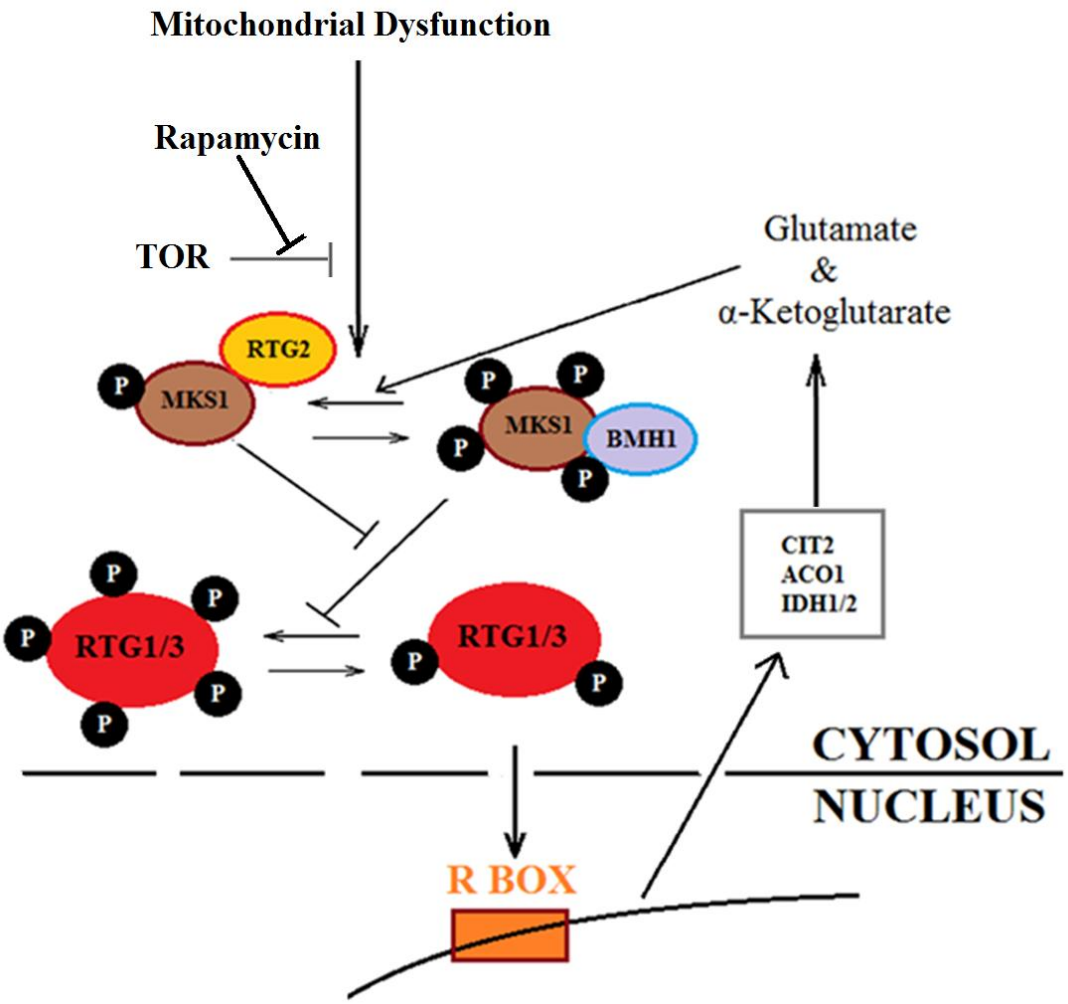


Figure 22. Proposed model for retrograde signaling activation in response to glutamate

CHAPTER V

SUMMARY

In this project, we aimed to investigate the functions of Rtg2p in mitochondrial retrograde signaling. Instead of generating mutations in *RTG2,* we used fungal homologs of Rtg2p. Computational analysis of Rtg2p homologs among species showed that *C. glabrata, A. gossypii, K. lactis* and *V. polyspora* have putative Rtg2p proteins. We analyzed the expression of these fungal Rtg2p homologs in our *S. cerevisiae rtg2*Δ shuffle strain. We measured not only glutamate auxotrophy but also Cit2p and Aco1p levels as these changes are related to activation of retrograde signaling.

Our findings show that these fungal Rtg2p homologs are functional in transmitting the mitochondrial signal to the nucleus and in producing a cellular response. *C. glabrata* and *K. lactis* Rtg2p homologs were fully functional with regards to all cellular responses tested. *A. gossypii* functional activity was reduced due to the lack of expression; and *V. polyspora* Rtg2p homolog was able to rescue glutamate auxotrophy but had reduced levels of Cit2p and Aco1p expression.

We also analyzed the molecular interaction between Rtg2p homologs and Mks1p. Our results showed that all fungal Rtg2p homologs had reduced binding to Mks1p compared to WT. However, these mutants showed an increased interaction between Mks1p and Rtg2p under inducing conditions. In addition, expression of Rtg2p homologs resulted in an apparent increase in interaction between Mks1p and Bmh1p when

retrograde signaling was activated. This suggests that Rtg2p may have a role in influencing Mks1p-Bmh1p interaction. It is possible that an interaction between Rtg2p and Mks1p is required for promoting a molecular change in Mks1p that functions to prevent its inhibitory effects on Rtg1p/Rtg3p thereby activating the pathway.

Rtg2p homologs were also analyzed under several stress conditions. For all the stressors tested, cells showed no increased sensitivity to these conditions except for H_2O_2 addition. However, induction of retrograde signaling, or constitutive expression of Rtg2p, did not provide resistance to H_2O_2 treatment suggesting that retrograde signaling is not directly involved in oxidative stress defense mechanisms.

Elevated expression of Cit2p upon rapamycin treatment required both expression of Rtg2p and Mks1p suggesting that under these conditions the TOR and retrograde signaling pathways may have share signaling components.

CHAPTER VI

FUTURE DIRECTIONS

Analysis of the role of Rtg2p on Mks1p-Bmh1p interaction

Signaling communication between organelles are important for cellular adaptation to environmental stress conditions. Organisms have evolved their own signaling networks depending on their needs. Understanding mitochondrial retrograde signaling is key for work focusing on therapeutic studies on mitochondria-based diseases and drug development against pathogenic yeast. Compared to other organisms, retrograde signaling mechanism in *S. cerevisiae* is the best characterized mitochondrial communication pathway. However, in yeast the molecular basis of the signal transduction pathway is not complete. Our results bring to the forefront additional questions that need to be addressed including - " What impact, if any, does Rtg2p have on regulating Mks1p function specifically addressing modification that alter Mks1p-Bmh1p interaction?"- To this end, studies that generate mutations in *MKS1* can be used to elucidate the domains within *MKS1* related to Rtg2p function. Furthermore the Rtg2p homologs characterized in this study can be used to identify the domains responsible for transmitting the retrograde signal from dysfunctional mitochondria to the nucleus.

Role of Rtg2p in mitochondrial retrograde signaling

Rtg2p has a well characterized role in the expression of mitochondrial and peroxisomal proteins via Rtg1p/Rtg3p dependent activation. However studies, including ours, have presented evidence for the involvement of Rtg2p in other signaling pathways.

For example, Chen and colleagues have shown that the defective colony formation at elevated temperature for the *crd1*Δ mutant (cardiolipin synthase mutant) can be rescued by deletion of *RTG2* but not deletion of *RTG3* [91]. This data suggest that, Rtg2p may be involved in another pathway to controls regulation of cardiolipin biosynthesis.

An interesting finding by Grant and colleagues showed that Rtg2p localizes to the nucleus along with the SLIK (SAGA-Like) complex. Both SLIK and SAGA are involved in histone acetylation but SLIK differs from SAGA by a few subunits [92-93]. It is known that 10% of genes are controlled by the SAGA complex. However, the exact function of SLIK and SAGA is unclear, as the genome-wide expression profile differences between the two complexes has yet to be determined [94]. It has been shown that Rtg2p is required for the integrity of the SLIK complex, and it was suggested that Rtg2p/SLIK complex is involved in the transcriptional regulation mediated by Rtg1p/Rtg3p [93]. However, it is not clear whether the association of Rtg2p with the SLIK complex is regulated by the level of Rtg2p localized to the nucleus. Preliminary data from our lab has found that Rtg2p levels are not influenced by glutamate suggesting that the interaction of Rtg2p with the SLIK complex is potentially controlled at a posttranslational level possibly affecting the association of Rtg2p with SLIK. In addition,

we have also detected Mks1p in the nucleus which was also independent of glutamate levels. Note however that localization of Mks1p to the nucleus can be attributed to its involvement in other pathways and does not necessarily mean that nuclear localized Mks1p has a function in retrograde signaling.

To identify the function of Rtg2p on SLIK complex formation and its impact on genome wide expression profiles, mutant strains can be generated that are incompetent for SLIK complex activity.

Identification of novel protein products that are controlled by mitochondrial retrograde signaling and TOR

Several genes have been identified as being under the control of the retrograde signaling pathway. However, recent findings on the connection of TOR related GATA factors and Rtg1p/Rtg3p suggest that additional genes may be regulated by these transcription factors. A bioinformatics software program has been developed to identify putative genes housing conserved transcription factor binding motifs (Figure 22) (Appendix for the Perl code). This tool can be used to search for R box and/or GATA box motifs in whole yeast genome and genes identified by this program can be analyzed using standard molecular biology techniques for biological confirmation of computational results.

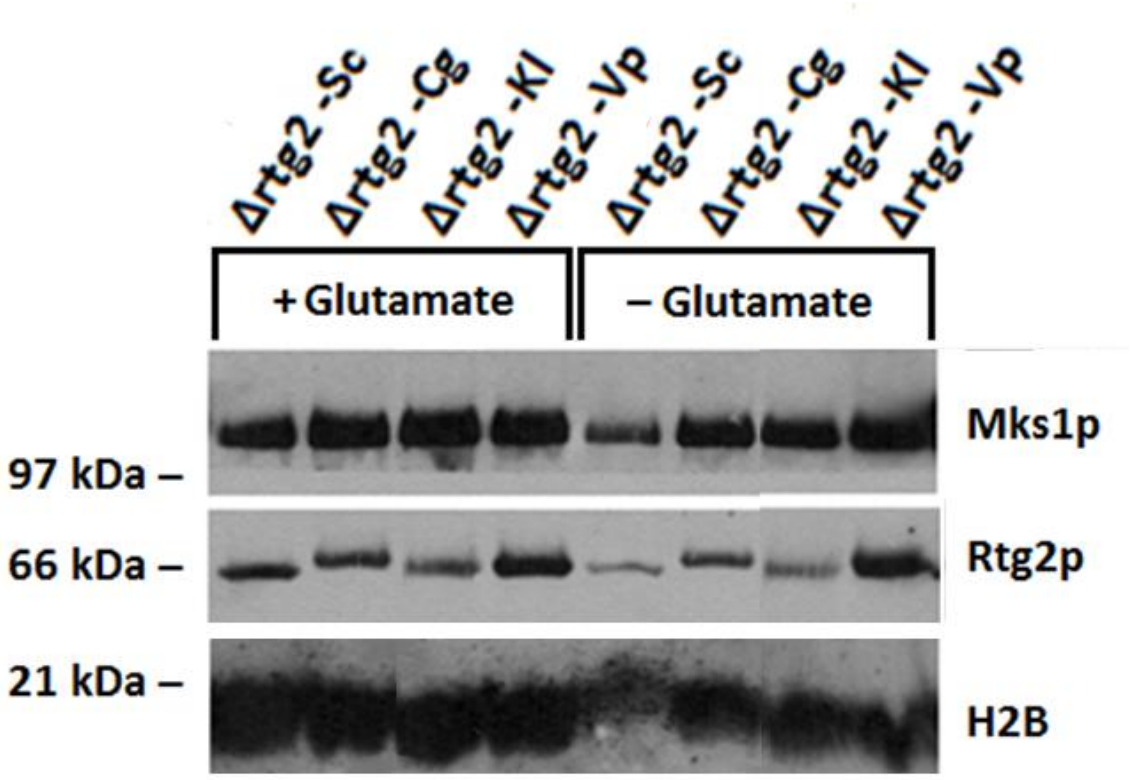


Figure 23. Nuclear localization profile of Rtg2p and Mks1p

Nuclear fractions were prepared from cells grow in the presence or absence of glutamate. Protein samples were processed by SDS-PAGE followed by western blot analysis using antibodies against Histone H2B, Myc to detect Mks1p, and HA to detect Rtg2p. H2B levels were used as a control for equal loading.

Figure 24. Interface of yeast promoter motif browser software

The software was written in Perl/Tk and uses FSA format chromosomal input files and FASTA format open reading frame sequence templates. These input files were downloaded from *S. cerevisiae* Genome Database (SGD) website and modified for the ease of presentation and faster data processing.

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APPENDIX A

PERL CODE FOR SERIAL CODON USAGE ANALYZER

```
#!/usr/local/bin/perl

print "\n\n\n \UPlease enter the directory where your files are stored\n\n";
chomp ($directory = <STDIN>);
print "\n\n\n \UPlease enter the file extension\n\n";
chomp ($extension = <STDIN>);
(open OUTFILEDIR, ">directory.txt") || (die "opening of directory.txt unsuccesfull");
opendir(DIR,$directory);
my @files = readdir(DIR);
closedir(DIR);
foreach(@files){
        if ($_ =~ /.$extension/){
  print OUTFILEDIR $_,"\n";
}
}
close OUTFILEDIR;
(open INFILE1, "directory.txt") || (die "opening of $chrfiles unsuccesfull");
while (@filenames = <INFILE1>){
        foreach $filename (@filenames){
(open INFILE2, $filename) || (die "opening of $filename unsuccesfull");
 (open INFILECODON, "codon1.txt") || (die "opening of codon1.txt unsuccessful");
(open INFILECODON2, "codon3.txt") || (die "opening of codon3.txt unsuccessful");
$outputfile = "output".$filename;
(open OUTFILE, ">$outputfile") || (die "opening of output.txt unsuccessful");
$genomic = <INFILE2>;
while ($genomic){
        $header = $genomic;
        $seq = '';
        do {
                chomp ($genomic = <INFILE2>);
                if ((defined ($genomic)) && (! ($genomic =~ /^>/))){
                        $genomic =~ s/\s//g;
                        $seq = $seq.$genomic;
                }
        }until ((! $genomic) || ($genomic =~ /^>/)) ;
                $sequences[$i] = $seq; #gets the sequence for each gene
        $size[$i] = length $sequences[$i]; #calculates the length of each gene
   $total += $size[$i]; #calculates the total length of the genes
   $seq=~ tr/tT/UU/;
   for (my $j=0; $j <= ($size[$i]-1); $j += 3){
```

```
    my $codoninputs = substr($seq, $j, 3);
        if (exists $triblets{$codoninputs}){
            $triblets{$codoninputs}++;
    }else{
            $triblets{$codoninputs} = 1;
    }
 }
$i++;
}
 %codons = (
       # A - Alanine
       'GCU'=>'A',
       'GCC'=>'A',
       'GCA'=>'A',
       'GCG'=>'A',
       # C - Cysteine
       'UGU'=>'C',
       'UGC'=>'C',
       # D - Aspartic Acid
       'GAU'=>'D',
       'GAC'=>'D',
       # E - Glutamic Acid
       'GAA'=>'E',
       'GAG'=>'E',
       # F - Phenylalanine
       'UUU'=>'F',
       'UUC'=>'F',
       # G - Glycine
       'GGU'=>'G',
       'GGC'=>'G',
       'GGA'=>'G',
       'GGG'=>'G',
       # H - Histidine
       'CAU'=>'H',
       'CAC'=>'H',
       # I - Isoleucine
       'AUU'=>'I',
       'AUC'=>'I',
       'AUA'=>'I',
       # K - Lysine
       'AAA'=>'K',
       'AAG'=>'K',
       # L - Leucine
       'CUU'=>'L',
```

```
'CUC'=>'L',
'CUA'=>'L',
'CUG'=>'L',
'UUA'=>'L',
'UUG'=>'L',
# M - Methionine
'AUG'=>'M',
# N - Asparagine
'AAU'=>'N',
'AAC'=>'N',
# P - Proline
'CCU'=>'P',
'CCC'=>'P',
'CCA'=>'P',
'CCG'=>'P',
# Q - Glutamine
'CAA'=>'Q',
'CAG'=>'Q',
# R - Arginine
'CGU'=>'R',
'CGC'=>'R',
'CGA'=>'R',
'CGG'=>'R',
'AGA'=>'R',
'AGG'=>'R',
# S - Serine
'UCU'=>'S',
'UCC'=>'S',
'UCA'=>'S',
'UCG'=>'S',
'AGU'=>'S',
'AGC'=>'S',
# T - Threonine
'ACU'=>'T',
'ACC'=>'T',
'ACA'=>'T',
'ACG'=>'T',
# V - Valine
'GUU'=>'V',
'GUC'=>'V',
'GUA'=>'V',
'GUG'=>'V',
# W - Tryptophan
'UGG'=>'W',
```

```
	# Y - Tyrosine
	'UAU'=>'Y',
	'UAC'=>'Y',
	# . - Stop
	'UAA'=>'STOP',
	'UAG'=>'STOP',
	'UGA'=>'STOP',
);
@codon = <INFILECODON>;
@codon2 = <INFILECODON2>;
chomp @codon;
chomp @codon2;
$z = 0;
for ($l=0; $l<=$#codon; $l++){
		while ($codons{$codon[$l]} eq $codons{$codon[$z]}) {
			$sum{$codon[$l]} += $triblets{$codon[$z]};
		$z++;
}
}
for ($k=0; $k<=$#codon; $k++){

		if (($sum{$codon2[$k]} > 0) && ($triblets{$codon[$k]} > 0)){
		print OUTFILE $codon[$k], "\t", $codons{$codon[$k]}, "\t",
$triblets{$codon[$k]},"\t",(($triblets{$codon[$k]}/$sum{$codon2[$k]})*100),"\n";
		}   elsif (($sum{$codon2[$k]} > 0) && ($triblets{$codon[$k]} = 0)){
		print OUTFILE $codon[$k], "\t", $codons{$codon[$k]}, "\t",
"0","\t",(($triblets{$codon[$k]}/$sum{$codon2[$k]})*100),"\n";
		}	else{
			print OUTFILE $codon[$k], "\t", $codons{$codon[$k]}, "\t",
$triblets{$codon[$k]},"\t","ZERO DIVISION ERROR","\n";
		}
}
@codon = "";
@codons = "";
%codon = "";
%triblets = "";
%sum = "";
@codon2 = "";
}
}
```

APPENDIX B

PERL CODE FOR YEAST PROMOTER MOTIF BROWSER

```
#!/usr/local/bin/perl

use Tk;
use Tk::ProgressBar;
use Tk::ROText;
my $mw = new MainWindow;

#MAINWINDOW
$mw->geometry("675x450");
$mw->title("YeaPROM");
$frame2 = $mw->Frame(-borderwidth => 2, -relief => 'groove')->place(-x=>1000,-y=>500);

$title = $mw -> Label(-text=>"YEAST PROMOTER MOTIF BROWSER", -font => ['courier',
'18', 'bold']) -> place(-relx => 0.2,
-rely => 0.0);

#CHROMOSOME SELECTION
$titleCHR = $mw -> Label(-text=>"CHROMOSOMES", -font => ['courier', '12', 'bold']) -
>place(-x=>50,-y=>80);

# ************************************************
$selectall = $mw->Checkbutton(-text => "Select All",
                              -command=> \&selectall,
                             )->place(-x=>50,-y=>100);

$clearall = $mw->Checkbutton(-text => "Clear All",
                                 -onvalue => "yes",
                                 -command=> \&clearall,
                                 -offvalue => "no",
                             )->place(-x=>150,-y=>280);
   sub selectall {
         $clearall ->deselect();
         $chkI ->select();
         $chkII ->select();
         $chkIII ->select();
         $chkIV ->select();
         $chkV ->select();
         $chkVI ->select();
         $chkVII ->select();
         $chkVIII ->select();
         $chkIX ->select();
         $chkX ->select();
         $chkXI ->select();
```

```
	$chkXII ->select();
	$chkXIII ->select();
	$chkXIV ->select();
	$chkXV ->select();
	$chkXVI ->select();
	$chkM ->select();

	}
sub clearall {
	$selectall ->deselect();
	$chkI ->deselect();
	$chkII ->deselect();
	$chkIII ->deselect();
	$chkIV ->deselect();
	$chkV ->deselect();
	$chkVI ->deselect();
	$chkVII ->deselect();
	$chkVIII ->deselect();
	$chkIX ->deselect();
	$chkX ->deselect();
	$chkXI ->deselect();
	$chkXII ->deselect();
	$chkXIII ->deselect();
	$chkXIV ->deselect();
	$chkXV ->deselect();
	$chkXVI ->deselect();
	$chkM ->deselect();
	$clearall ->deselect();
}
# ****************************************************
$chkI = $mw->Checkbutton(-text => "I",

	-onvalue => "yes",
	-variable => \$chr1,
	-offvalue => "no",
)->place(-x=>50,-y=>120);
# ****************************************************
$chkII = $mw->Checkbutton(-text => "II",
			-onvalue => "yes",
			-variable => \$chr2,
			-offvalue => "no",
			)->place(-x=>50,-y=>140);
# ****************************************************
$chkIII = $mw->Checkbutton(-text => "III",

	-onvalue => "yes",
	-variable => \$chr3,
	-offvalue => "no",
```

```
)->place(-x=>50,-y=>160);
# ***************************************************
$chkIV = $mw->Checkbutton(-text => "VI",

      -onvalue => "yes",
      -variable => \$chr4,
      -offvalue => "no",
)->place(-x=>50,-y=>180);
# ***************************************************
$chkV = $mw->Checkbutton(-text => "V",

      -onvalue => "yes",
      -variable => \$chr5,
      -offvalue => "no",
)->place(-x=>50,-y=>200);
# ***************************************************
$chkVI = $mw->Checkbutton(-text => "VI",

      -onvalue => "yes",
      -variable => \$chr6,
      -offvalue => "no",
)->place(-x=>50,-y=>220);
# ***************************************************
$chkVII = $mw->Checkbutton(-text => "VII",

      -onvalue => "yes",
      -variable => \$chr7,
      -offvalue => "no",
)->place(-x=>50,-y=>240);
# ***************************************************
$chkVIII = $mw->Checkbutton(-text => "VIII",

      -onvalue => "yes",
      -variable => \$chr8,
      -offvalue => "no",
)->place(-x=>50,-y=>260);
# ***************************************************
$chkIX = $mw->Checkbutton(-text => "IX",

      -onvalue => "yes",
      -variable => \$chr9,
      -offvalue => "no",
)->place(-x=>50,-y=>280);
# ***************************************************
$chkX = $mw->Checkbutton(-text => "X",

      -onvalue => "yes",
      -variable => \$chr10,
```

```
	-offvalue => "no",
)->place(-x=>150,-y=>120);
# ***************************************************
$chkXI = $mw->Checkbutton(-text => "XI",

	-onvalue => "yes",
	-variable => \$chr11,
	-offvalue => "no",
)->place(-x=>150,-y=>140);

# ***************************************************
$chkXII = $mw->Checkbutton(-text => "XII",

	-onvalue => "yes",
	-variable => \$chr12,
	-offvalue => "no",
)->place(-x=>150,-y=>160);
# ***************************************************
$chkXIII = $mw->Checkbutton(-text => "XIII",

	-onvalue => "yes",
	-variable => \$chr13,
	-offvalue => "no",
)->place(-x=>150,-y=>180);
# ***************************************************
$chkXIV = $mw->Checkbutton(-text => "XIV",

	-onvalue => "yes",
	-variable => \$chr14,
	-offvalue => "no",
)->place(-x=>150,-y=>200);
# ***************************************************
$chkXV = $mw->Checkbutton(-text => "XV",

	-onvalue => "yes",
	-variable => \$chr15,
	-offvalue => "no",
)->place(-x=>150,-y=>220);
# ***************************************************
$chkXVI = $mw->Checkbutton(-text => "XVI",

	-onvalue => "yes",
	-variable => \$chr16,
	-offvalue => "no",
)->place(-x=>150,-y=>240);
# ***************************************************
$chkM = $mw->Checkbutton(-text => "Mitochondria",
```

```
	-onvalue => "yes",
	-variable => \$chrM,
	-offvalue => "no",
)->place(-x=>150,-y=>260);

# MOTIF ENTRY

$MOT = $mw -> Label(-text=>"MOTIF:", -font => ['courier', '10', 'bold']) ->place(-x=>350,-
y=>80);
$ent1 = $mw->Entry(-text=>"MOTIF",-cursor => "", -textvariable => \$domainmotif, -
width=>22) -> place(-x=>405,-y=>80);
$butM = $mw -> Button(-text=>"?",-font => ['', '9', 'bold'],-relief=>groove , -command
=>\&helpmotif);
$butM -> place(-x=>555,-y=>75);

# **************Motif Entry Help **************
sub helpmotif{
$topM = $mw -> Toplevel();
$topM->geometry("500x200");
$topM ->title('MOTIF ENTRY HELP');


$txtM1 =  $topM ->Label(-text=>"A: Adenine", -font => ['courier', '10', 'bold'])->place(-x=>20,-
y=>10);
$txtM2 =  $topM ->Label(-text=>"T: Thymine", -font => ['courier', '10', 'bold'])->place(-x=>20,-
y=>30);
$txtM3 =  $topM ->Label(-text=>"G: Guanine", -font => ['courier', '10', 'bold']) ->place(-x=>20,-
y=>50);
$txtM4 =  $topM ->Label(-text=>"C: Cytosine", -font => ['courier', '10', 'bold']) ->place(-x=>20,-
y=>70);
$txtM5 =  $topM ->Label(-text=>"N: Any nucleotide (A or T or G or C)", -font => ['courier', '10',
'bold']) ->place(-x=>20,-y=>90);
$txtM6 =  $topM ->Label(-text=>"R: Purines (G or A)", -font => ['courier', '10', 'bold']) ->place(-
x=>20,-y=>110);
$txtM7 =  $topM ->Label(-text=>"Y: Pyrimidines (T or C)", -font => ['courier', '10', 'bold']) -
>place(-x=>20,-y=>130);
$txtM8 =  $topM ->Label(-text=>"Example: ATCGRC => Motif will be searched as ATCG-
G/A-C ", -font => ['courier', '10', italic]) ->place(-x=>20,-y=>150);
$but_closeM = $topM -> Button(-text=>"Close",
                    -command => sub { destroy $topM; } ) ->place(-x=>240,-y=>173);

}

#STRAND SELECTION: WATSON OR CRICK

$strand = "W";
$chk1 = $mw->Checkbutton(-text => "Watson",
                                -onvalue => "W",
```

```
                                -variable => \$strand,
                                -offvalue => "",
                            )->place(-x=>413,-y=>130);

$chk2 = $mw->Checkbutton(-text => "Crick",
                                -onvalue => "C",
                                -variable => \$strand,
                                -offvalue => "",
                            )->place(-x=>490,-y=>130);

# SETTING THE SEARCH WINDOW

$uplimit = 1000;
$lowlimit = 1;
$PRANGE = $mw -> Label(-text=>"SEARCH WINDOW", -font => ['courier', '12', 'bold']) -
>place(-x=>410,-y=>180);
$butSW = $mw -> Button(-text=>"?",-font => ['', '9', 'bold'],-relief=>groove , -command
=>\&helpSW);
$butSW -> place(-x=>555,-y=>180);

# **************Search Window Help **************
sub helpSW{
$topSW = $mw -> Toplevel();
$topSW->geometry("850x155");
$topSW ->title('SEARCH WINDOW HELP');

$UPRANGESW2 = $topSW -> Label(-text=>"5'----", -font => ['courier', '10', 'bold']) ->place(-
x=>20,-y=>10);
$upentSW2 = $topSW->Entry(-text => "1000",-background => grey,-state => 'disabled', -
width=>5) -> place(-x=>76,-y=>10);
$MIDLINESW2 = $topSW -> Label(-text=>"----", -font => ['courier', '10', 'bold']) ->place(-
x=>114,-y=>10);
$lowentSW2 = $topSW->Entry(-text => "1",  -width=>5) -> place(-x=>152,-y=>10);
$LOWRANGESW2 = $topSW -> Label(-text=>"----ATG-3'", -font => ['courier', '10', 'bold']) -
>place(-x=>190,-y=>10);
$textdownSW = $topSW -> Label(-text=>"Right Box: Sets the closest nucleotide position to
transcriptional start codon to start the search", -font => ['courier', '10', 'bold']) ->place(-x=>20,-
y=>30);

$UPRANGESW = $topSW -> Label(-text=>"5'----", -font => ['courier', '10', 'bold']) ->place(-
x=>20,-y=>85);
$upentSW = $topSW->Entry(-text => "1000", -width=>5) -> place(-x=>76,-y=>85);
$MIDLINESW = $topSW -> Label(-text=>"----", -font => ['courier', '10', 'bold']) ->place(-
x=>114,-y=>85);
$lowentSW = $topSW->Entry(-text => "1",-background => grey, -state => 'disabled', -width=>5)
-> place(-x=>152,-y=>85);
$LOWRANGESW = $topSW -> Label(-text=>"----ATG-3'", -font => ['courier', '10', 'bold']) -
>place(-x=>190,-y=>85);
```

```
$textupSW = $topSW -> Label(-text=>"Left Box: Sets the far most nucleotide position from transcriptional start codon to stop the search", -font => ['courier', '10', 'bold']) ->place(-x=>20,-y=>105);

$but_closeSW = $topSW -> Button(-text=>"Close",
                    -command => sub { destroy $topSW; } ) -> place(-x=>400,-y=>128);
}
# ***************************************************
$UPRANGE = $mw -> Label(-text=>"5'----", -font => ['courier', '10', 'bold']) ->place(-x=>350,-y=>220);
$upent = $mw->Entry(-textvariable => \$uplimit, -width=>5) -> place(-x=>406,-y=>220);
$MIDLINE = $mw -> Label(-text=>"----", -font => ['courier', '10', 'bold']) ->place(-x=>444,-y=>220);
$lowent = $mw->Entry(-textvariable => \$lowlimit, -width=>5) -> place(-x=>482,-y=>220);
$LOWRANGE = $mw -> Label(-text=>"----ATG-3'", -font => ['courier', '10', 'bold']) ->place(-x=>520,-y=>220);

#SELECTION OF THE MOTIF ORIENTATION

$titleDORIENT =  $mw -> Label(-text=>"ORIENTATION", -font => ['courier', '12', 'bold']) ->place(-x=>420,-y=>260);

$domainsearch = "S";
$chkA = $mw->Checkbutton(-text => "ANTISENSE",

                                -onvalue => "S",
                                -variable => \$domainsearch,
                                -offvalue => "None",
                            )->place(-x=>325,-y=>300);

$chkS = $mw->Checkbutton(-text => "SENSE",

                                -onvalue => "O",
                                -variable => \$domainsearch,
                                -offvalue => "None",
                            )->place(-x=>428,-y=>300);
$chkIR = $mw->Checkbutton(-text => "INVERTED REPEAT",

                                -onvalue => "P",
                                -variable => \$domainsearch,
                                -offvalue => "None",
                            )->place(-x=>506,-y=>300);

# **************Motif Orientation Help **************
$butO = $mw -> Button(-text=>"?",-font => ['', '9', 'bold'],-relief=>groove , -command =>\&helporientation);
$butO -> place(-x=>555,-y=>260);
```

```
sub helporientation{
$topO = $mw -> Toplevel();
$topO ->title('ORIENTATION HELP');

$txtO1 =  $topO ->Label(-text=>"Select orientation of the motif with respect to transcriptional
start site", -font => ['courier', '10', 'bold']) -> pack;

$txtO2 =  $topO ->Label(-text=>"  ANTISENSE: Search the motif in the same strand with
respect to transcriptional start", -font => ['courier', '8', 'bold']) -> pack;

$txtO3 =  $topO ->Label(-text=>"   SENSE: Search the motif in complementary strand with
respect to transcriotional start", -font => ['courier', '8', 'bold']) -> pack;

$txtO2 =  $topO ->Label(-text=>"INVERTED REPEAT: Search the motif in both strands which
exists as a palindrom form", -font => ['courier', '8', 'bold']) -> pack;
$but_closeO = $topO -> Button(-text=>"Close",
                    -command => sub { destroy $topO; } ) -> pack;

}
# ***************************************************
$but = $mw -> Button(-text=>"Start", -command =>\&startbuffer);
$but -> place(-x=>450,-y=>350);

#DISPLAY OF RESULTS

sub toplevel{
$top = $mw -> Toplevel();
$top->geometry("750x500");
$sepro = "SEARCH IN PROGRESS";
$top_lab = $top -> Label(-text=>"$sepro",
                                -font=>"ansi 12 bold") -> place(-x=>265,-y=>20);
                                $tab = sprintf("%-7s %-30s %-30s\n", "#", "Hits      Position(s)",
"\t\tGene Name and Description");


$top_tab = $top -> Label(-text=>"$tab",
                                -font=>"ansi 10 bold") -> place(-x=>20,-y=>50);

$txt = $top->Scrolled(
                    'ROText',
                    -scrollbars => 'se',
                    -width => 100,
                    -height => 15,
                    )->place(-x=>17,-y=>70);

sub results {

   $txt->insert('end', sprintf "$results %s\n");
```

```
  $txt->see('end');
}
$but_close = $top -> Button(-text=>"Close",
                    -command => sub { exit } ) -> place(-x=>350,-y=>475);


$text = "Percentage finished: $counter";
$top->title('Progress');
$message = $top->Message(-textvariable => \$text,
                     -width => 200,
                     -border => 2);
$progress = $top->ProgressBar(-width => 50,
                                        -height => 30,
                -from => 0,
                -to => 100,
                -anchor => 'w',
                -blocks => 50,
                -colors => [0, 'grey'],
                -variable => \$counter,
                );
$progress->place(-x=>175,-y=>400);
$message->place(-x=>310,-y=>430);

}

MainLoop;
# BUFFER LOOOP STARTS
sub startbuffer {
          $score1 = 0;
          $score2 = 0;
          $score = 0;

          if ( ($chr1 ne "yes")&&( $chr2 ne "yes")&&( $chr3 ne "yes")&&( $chr4 ne "yes")
          &&( $chr5 ne "yes")&&( $chr6 ne "yes")&&( $chr7 ne "yes")&&( $chr8 ne "yes")
          &&( $chr9 ne "yes")&&( $chr10 ne "yes")&&( $chr11 ne "yes")&&( $chr12 ne "yes")
          &&( $chr13 ne "yes")&&( $chr14 ne "yes")&&( $chr15 ne "yes")&&( $chr16 ne
"yes")&&( $chrM ne "yes")) {
                    $score0 = 1;
                    $mw->withdraw();
          $topbuffer0 = $mw -> Toplevel();

          $topbuffer0 ->title('!!!SOMETHING IS WRONG :( ');


$txtB0 =  $topbuffer0 ->Label(-text=>"SELECT AT LEAST ONE CHROMOSOME.PLEASE
RE-ENTER AGAIN", -font => ['courier', '10', 'bold']) -> pack;
$but_buffer0 = $topbuffer0 -> Button(-text=>"Close",
                    -command => sub { destroy $topbuffer0;$mw->deiconify(); } ) -> pack;
```

```
}
        $domainmotif =~ s/\s//gi;
        if ($domainmotif =~ /([^ATCGNRY|()])/gi){
                $mw->withdraw();
        $score1 = 1;
        $topbuffer1 = $mw -> Toplevel();

        $topbuffer1 ->title('!!!SOMETHING IS WRONG :( ');
        $txtB1 =  $topbuffer1 ->Label(-text=>"THERE IS INVALID LETTER IN YOUR
MOTIF. PLEASE RE-ENTER AGAIN", -font => ['courier', '10', 'bold']) -> pack;
        $but_buffer1 = $topbuffer1 -> Button(-text=>"Close",
                -command => sub { destroy $topbuffer1;$mw->deiconify(); } ) -> pack;
}
         if ($uplimit <= $lowlimit){
           $mw->withdraw();
           $score2 = 1;
           $topbuffer2 = $mw -> Toplevel();

        $topbuffer2 ->title('!!!SOMETHING IS WRONG :( ');
        $txtB2 =  $topbuffer2 ->Label(-text=>"SUM OF SEARCH WINDOW CANNOT BE
NEGATIVE. PLEASE RE-ENTER AGAIN", -font => ['courier', '10', 'bold']) -> pack;
        $but_buffer2 = $topbuffer2 -> Button(-text=>"Close",
                -command => sub { destroy $topbuffer2;$mw->deiconify(); } ) -> pack;
}
$score = ($score0 + $score1 + $score2);
if ($score <1){
&start;
} else{ $score0 = 0; $score1 = 0; $score2 = 0;$mw->update();
}

}

#*************************START POINT*************************
sub start{
                $found = 1;

  if ( $chr1 eq "yes" ) { push @datasets, "chr1.fsa";}
  if ( $chr2 eq "yes" ) { push @datasets, "chr2.fsa";}
  if ( $chr3 eq "yes" ) { push @datasets, "chr3.fsa";}
  if ( $chr4 eq "yes" ) { push @datasets, "chr4.fsa";}
  if ( $chr5 eq "yes" ) { push @datasets, "chr5.fsa";}
  if ( $chr6 eq "yes" ) { push @datasets, "chr6.fsa";}
  if ( $chr7 eq "yes" ) { push @datasets, "chr7.fsa";}
  if ( $chr8 eq "yes" ) { push @datasets, "chr8.fsa";}
  if ( $chr9 eq "yes" ) { push @datasets, "chr9.fsa";}
  if ( $chr10 eq "yes" ) { push @datasets, "chr10.fsa";}
  if ( $chr11 eq "yes" ) { push @datasets, "chr11.fsa";}
  if ( $chr12 eq "yes" ) { push @datasets, "chr12.fsa";}
```

```
  if ( $chr13 eq "yes" ) { push @datasets, "chr13.fsa";}
  if ( $chr14 eq "yes" ) { push @datasets, "chr14.fsa";}
  if ( $chr15 eq "yes" ) { push @datasets, "chr15.fsa";}
  if ( $chr16 eq "yes" ) { push @datasets, "chr16.fsa";}
  if ( $chrM eq "yes" ) { push @datasets, "chrmt.fsa";}

&toplevel;

$top->deiconify();

        $mw->withdraw();
$counter = 0;
$counterplus = 100/($#datasets + 1);
foreach $dataset (@datasets){
chomp $dataset;
$namefile = $dataset;
(open INFILE, "$dataset") || (die "opening of $dataset unsuccessful");

if ( $dataset eq "chr1.fsa" ) {$s = 0;$st = 93; }
if ( $dataset eq "chr2.fsa" ) {$s = 93;$st = 500;}
if ( $dataset eq "chr3.fsa" ) {$s = 500;$st = 660;}
if ( $dataset eq "chr4.fsa" ) {$s = 660;$st = 1414;}
if ( $dataset eq "chr5.fsa" ) {$s = 1414;$st = 1691;}
if ( $dataset eq "chr6.fsa" ) {$s = 1691;$st = 1817;}
if ( $dataset eq "chr7.fsa" ) {$s = 1817;$st = 2344;}
if ( $dataset eq "chr8.fsa" ) {$s = 2344;$st = 2625;}
if ( $dataset eq "chr9.fsa" ) {$s = 2625;$st = 2832;}
if ( $dataset eq "chr10.fsa" ) {$s = 2832;$st = 3189;}
if ( $dataset eq "chr11.fsa" ) {$s = 3189;$st = 3501;}
if ( $dataset eq "chr12.fsa" ) {$s = 3501;$st = 4009;}
if ( $dataset eq "chr13.fsa" ) {$s = 4009;$st = 4469;}
if ( $dataset eq "chr14.fsa" ) {$s = 4469;$st = 4862;}
if ( $dataset eq "chr15.fsa" ) {$s = 4862;$st = 5398;}
if ( $dataset eq "chr16.fsa" ) {$s = 5398;$st = 5862;}
if ( $dataset eq "chrmt.fsa" ) {$s = 5862;$st = 5881;}

$text = "Percentage Finished: $counter";
   $top->update;
   sleep 1;
$counter = $counter + $counterplus;
$counter = sprintf("%.0f", $counter);
$i = 0;
@names =";
@sequences = ";
$sctotal =";
@scgenome = <INFILE>;
chomp @scgenome;
close INFILE;
```

```
@sequences = '';

foreach $scgenome (@scgenome){
	$sctotal .= $scgenome;
}
@scgenome = '';
$scgenomerev = reverse $sctotal;
$sctotal = '';
# ***************************************************
(open ORFFILE2, "names.txt") || (die "unsuccesfull");
chomp (@names = <ORFFILE2>);

close ORFFILE2;
(open ORFFILE5, "sequences.txt") || die ("opening of sequences.txt unsuccesfull");
@sequences = <ORFFILE5>;
close ORFFILE5;
# ***************************************************
chomp $domainmotif;
$domainmotif = uc $domainmotif;
$domain = $domainmotif;
if ($domain =~ /N/){
	$domain =~ s/N/(A|T|G|C)/g;
}
if ($domain =~ /R/){
	$domain =~ s/R/(A|G)/g;
}
if ($domain =~ /Y/){
	$domain =~ s/Y/(T|C)/g;
}

$domain1 = reverse $domain;
$domain1 =~ tr/)(/()/;
$domain2 = reverse $domain1;
$domain2 =~ tr/)(/()/;
$domain2 =~ tr/atcgATCG/tagcTAGC/;
$domain3 = reverse $domain2;
$domain3 =~ tr/)(/()/;
# ***************************************************#
chomp $lowlimit;
$lowlimit = ($lowlimit-1);
chomp $uplimit;
# ***************************************************
chomp $strand;
chomp $domainsearch;

#SEARCH STARTS

if ($strand eq 'W'){ #Search for Watson Strand-Loop Start
```

```
if ($domainsearch eq 'P'){
	$domainsearch1 = "DOMAIN SEARCH AS PALINDROM";
	goto PALINDROM;
}	elsif ($domainsearch eq 'O'){
	$domainsearch1 = "DOMAIN SEARCHED IN OPPOSITE STRAND";
	goto OPPOSITE;
}   else{
	$domainsearch1 = "DOMAIN SEARCHED IN SAME STRAND";
	goto SAME;
}
# ************************************************
	SAME: #Start domain search in same strand of which is used to detect promoter regions
(open ORFFILEN2, ">>ng1.txt") || (die "unsuccesfull");
(open ORFFILEN3, ">>seqs1.txt") || (die "unsuccesfull");

for ($i=$s; $i <= $st; $i++){
			chomp  $sequences[$i];
			chomp $names[$i];
			$sequences[$i] = reverse $sequences[$i];
			if ($scgenomerev =~
/($sequences[$i])[ATCG]{$lowlimit,$uplimit}($domain1)/){

	$match = (length $1)-(length $2);
 $scgenomerev =~ /(($sequences[$i])[ATCG]{$lowlimit,$uplimit})/;
$matchcnt = substr($1, 60, $uplimit);
$position = '';
while ($matchcnt =~ /$domain1/g){
($matchcnt =~ /(([ATCG]{0,1000})($domain1))/);
$matchcounter++;
$pos = pos($matchcnt) - ((length $domain1)-1);
$position .= "+".$pos."  ";
}
$position = $matchcounter."     ".$position;
$matchcounter = 0;
push @positions, $position;
			$nametrim = substr($names[$i], 7, 1);
				if ($nametrim =~ /C/){
					goto JUMP;
				}
				$results = sprintf("%-5s %-30s %-30s\n", "$found", "$position",
"$names[$i]");
				&results;
				push @results, $results;
				$found++;
			}
	JUMP:
			$top->update;
```

```
	}  #Search for SAME:Watson strand-Loop End
	close ORFFILEN2;
	close ORFFILEN3;
goto LAST;
# ************************************************
	OPPOSITE: #Start domain search in opposite strand which is opposite to strand that
promoter regions are detected.
	for ($i=$s; $i <= $st; $i++){
		chomp  $sequences[$i];
		chomp $names[$i];
		$sequences[$i] = reverse $sequences[$i];
		if (($scgenomerev =~
/($sequences[$i])([ATCG]{$lowlimit,$uplimit})($domain3)/)){
$match = (length $1)-(length $2);
 $scgenomerev =~ /(($sequences[$i])[ATCG]{$lowlimit,$uplimit})/;
$matchcnt = substr($1, 60, $uplimit);
$position = '';
while ($matchcnt =~ /$domain3/g){
($matchcnt =~ /(([ATCG]{0,1000})($domain3))/);
$matchcounter++;
$pos = pos($matchcnt) - ((length $domain3)-1);
$position .= "+".$pos."  ";
}
$position = $matchcounter."     ".$position;
push @positions, $position;
		$nametrim = substr($names[$i], 7, 1);
			if ($nametrim =~ /C/){
				goto JUMP;
			}
			$matchcounter = 0;
			$results = sprintf("%-5s %-30s %-30s\n", "$found", "$position",
"$names[$i]");
			&results;
			push @results, $results;
			$found++;
		}
	JUMP:
		$top->update;
	}  #Search for OPPOSITE:Watson strand-Loop End
goto LAST;
# ************************************************
	PALINDROM: #Start domain search in both strands to find palindromic form of domain
	for ($i=$s; $i <= $st; $i++){
		chomp  $sequences[$i];
		chomp $names[$i];
		$sequences[$i] = reverse $sequences[$i];
		if (($scgenomerev =~
/($sequences[$i])([ATCG]{$lowlimit,$uplimit})($domain2)([ATCG]{0,50})($domain1)/)||($scge
```

```
nomerev =~
/($sequences[$i])([ATCG]{$lowlimit,$uplimit})($domain1)([ATCG]{0,50})($domain2)/)){
                $match = (length $1)-(length $2);
 $scgenomerev =~ /(($sequences[$i])[ATCG]{$lowlimit,$uplimit})/;
$matchcnt = substr($1, 60, $uplimit);
$position = '';
while ($matchcnt =~ /$domain1/g){
($matchcnt =~ /(([ATCG]{0,1000})($domain1))/);
$matchcounter++;
$pos = pos($matchcnt) - ((length $domain1)-1);
$position .= "+".$pos."  ";
}
$position = $matchcounter."     ".$position;
$matchcounter = 0;
push @positions, $position;
$nametrim = substr($names[$i], 7, 1);
                        if ($nametrim =~ /C/){
                                goto JUMP;
                        }
                        $results = sprintf("%-5s %-30s %-30s\n", "$found", "$position",
"$names[$i]");
                        &results;
                        push @results, $results;
                        $found++;
                }
        JUMP:
                $top->update;
        }  #Search for Watson strand-Loop End
goto LAST;
# ***************************************************
} elsif ($strand eq 'C'){  #Search for Crick Strand-Loop Start

if ($domainsearch eq 'P'){ #Give the direction for searching the domain in terms of strand
        $domainsearch1 = "DOMAIN SEARCH AS PALINDROM";
        goto PALINDROM1;
}       elsif ($domainsearch eq 'O'){
        $domainsearch1 = "DOMAIN SEARCHED IN OPPOSITE STRAND";
        goto OPPOSITE1;
        }   else{
        $domainsearch1 = "DOMAIN SEARCHED IN SAME STRAND";
        goto SAME1;
}
# ***************************************************
                SAME1: #Start domain search in same strand of which is used to detect promoter
regions
(open ORFFILEN2, ">>ng1.txt") || (die "unsuccesfull");
(open ORFFILEN3, ">>seqs1.txt") || (die "unsuccesfull");
        $scgenomerev = reverse $scgenomerev;
```

```
	$scgenomerev =~ tr/atcgATCG/tagcTAGC/;
	for ($i=$s; $i <= $st; $i++){
		chomp  $sequences[$i];
		chomp $names[$i];
		$sequences[$i] = reverse $sequences[$i];
		if ($scgenomerev =~
/($sequences[$i])[ATCG]{$lowlimit,$uplimit}($domain1)/){
$match = (length $1)-(length $2);
 $scgenomerev =~ /(($sequences[$i])[ATCG]{$lowlimit,$uplimit})/;
$matchcnt = substr($1, 60, $uplimit);
$position = '';
while ($matchcnt =~ /$domain1/g){
($matchcnt =~ /(([ATCG]{0,1000})($domain1))/);
$matchcounter++;
$pos = pos($matchcnt) - ((length $domain1)-1);
$position .= "+".$pos."  ";
}
$position = $matchcounter."     ".$position;
$matchcounter = 0;
push @positions, $position;
		$nametrim= (substr ($names[$i], 7, 1));
			if ($nametrim =~ /W/){
				goto JUMP;
			}
			$results = sprintf("%-5s %-30s %-30s\n", "$found", "$position",
"$names[$i]");
			&results;
			push @results, $results;
			$found++;
		}
	JUMP:
		$top->update;
	} #Search for SAME:Crick strand-Loop End
	close ORFFILEN2;
	close ORFFILEN3;
goto LAST;
# ************************************************
		OPPOSITE1: #Start domain search in opposite strand which is opposite to strand
that promoter regions are detected.
	$scgenomerev = reverse $scgenomerev;
	$scgenomerev =~ tr/atcgATCG/tagcTAGC/;
	for ($i=$s; $i <= $st; $i++){
		chomp  $sequences[$i];
		chomp $names[$i];
		$sequences[$i] = reverse $sequences[$i];
		if (($scgenomerev =~
/($sequences[$i])([ATCG]{$lowlimit,$uplimit})($domain3)/)){
$match = (length $1)-(length $2);
```

```
 $scgenomerev =~ /(($sequences[$i])[ATCG]{$lowlimit,$uplimit})/;
$matchcnt = substr($1, 60, $uplimit);
$position = '';
while ($matchcnt =~ /$domain3/g){
($matchcnt =~ /(([ATCG]{0,1000})($domain3))/);
$matchcounter++;
$pos = pos($matchcnt) - ((length $domain3)-1);
$position .= "+".$pos."  ";
}
$position = $matchcounter."     ".$position;
$matchcounter = 0;
push @positions, $position;
                $nametrim= (substr ($names[$i], 7, 1));
                        if ($nametrim =~ /W/){
                                goto JUMP;
                        }
                        $results = sprintf("%-5s %-30s %-30s\n", "$found", "$position",
"$names[$i]");
                        &results;
                        push @results, $results;
                        $found++;
                }
        JUMP:
        $top->update;
        }  #Search for OPPOSITE:Crick strand-Loop End
goto LAST;
# ***************************************************
                PALINDROM1:  #Start domain search in both strands to find palindromic form
of domain
        $scgenomerev = reverse $scgenomerev;
        $scgenomerev =~ tr/atcgATCG/tagcTAGC/;
        for ($i=$s; $i <= $st; $i++){
        chomp  $sequences[$i];
        chomp $names[$i];
        $sequences[$i] = reverse $sequences[$i];
                if (($scgenomerev =~
/($sequences[$i])([ATCG]{$lowlimit,$uplimit})($domain2)([ATCG]{0,50})($domain1)/)||($scge
nomerev =~
/($sequences[$i])([ATCG]{$lowlimit,$uplimit})($domain1)([ATCG]{0,50})($domain2)/)){
                $match = (length $1)-(length $2);
 $scgenomerev =~ /(($sequences[$i])[ATCG]{$lowlimit,$uplimit})/;
$matchcnt = substr($1, 60, $uplimit);
$position = '';
while ($matchcnt =~ /$domain1/g){
($matchcnt =~ /(([ATCG]{0,1000})($domain1))/);
$matchcounter++;
$pos = pos($matchcnt) - ((length $domain1)-1);
$position .= "+".$pos."  ";
```

```
}
$position = $matchcounter."     ".$position;
$matchcounter = 0;
push @positions, $position;
                $nametrim= (substr ($names[$i], 7, 1));
                        if ($nametrim =~ /W/){
                                goto JUMP;
                        }
                        $results = sprintf("%-5s %-30s %-30s\n", "$found", "$position",
"$names[$i]");
                        &results;
                        push @results, $results;
                        $found++;
                }
        JUMP:
        $top->update;
        }  #Search for PALINDROM:Crick strand-Loop End
#Search for Crick strand-Loop End
}
# ***************************************************
LAST:
$lowlimit = ($lowlimit+1);
}
$text = '           Done!          ';
$sepro = "COMPLETED";
$tab_save = sprintf("%-4s %-29s %-30s\n", "#", "Hits      Position(s)", "\t\tGene Name and
Description");

$foundn = ($found -1);
$but_save = $top -> Button(-text=>"Save Results",
                -command => \&save ) ->place (-x=>332,-y=>450);
$top_lab ->update()
}
# ***************************************************
sub save {
        $but_save ->destroy;

        $dateandtime = localtime time;
        open (OUTPUT, ">>OUTPUT.txt") || (die "openin output.txt is unsuccesfull");

        print OUTPUT "DATE\t: $dateandtime\n\n";

        print OUTPUT "DOMAIN\t: $domain\n\n";
        print OUTPUT "# FOUND\t: $foundn\n\n";
        print OUTPUT "WINDOW\t:  5`------ $uplimit-----$lowlimit----ATG-3`\n\n";
        if ($strand eq "W"){
   print OUTPUT "STRAND\t: Watson\n\n\n";
        }elsif ($strand eq "C"){
```

```
	print OUTPUT "STRAND\t: Crick\n\n\n";

	}
	print OUTPUT $tab_save;
		$number = 1;
		foreach $result (@results){
		print OUTPUT $result;
		$number++;
	}

	print OUTPUT "\n-----------------------------------------------------------------\n\n\n";
	close OUTPUT;
	$text = 'Results are saved into OUTPUT.TXT';
}

##################### THIS IS THE END ####################
```